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Genetic and phenotypic evidence suggest the existence of indigenous olive population of wild var. *sylvestris* in the Carmel coast, southern Levant

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Abstract

Background Populations of *Olea europaea* subsp. *europaea* var. *sylvestris*, the ancestor of cultivated olives, are scattered across the Mediterranean Basin. However, after millennia of possible hybridization with cultivated varieties, the genetic identity of many of these populations remain questionable. In the southern Levant, the plausible primary domestication center of olives, many of the naturally growing olive (NGOs) are considered feral, having developed from nearby olive groves. Here, we investigated the genetic identity of NGOs population in the Carmel region, hypothesizing that their specific location, which limit anemophily, provided an opportunity for the persistence of genuine var. *sylvestris*.

Results We mapped more than 1,000 NGOs on the Kurkar ridge along the Carmel coast, within and outside the residential area of Atlit and used simple sequence repeats of 14 loci to assess the spatial genetic structure of 129 NGOs. Genetic diversity parameters and genetic distances between NGO and cultivated olives, as well as phenotypic and morphometric analyses of their oil content and pits, respectively, indicated the presence of a genuine var. *sylvestris* population. However, NGOs within the residential area of Atlit and old settlements showed an intermediate admix genetic structure, indicating on hybridization with local varieties, a consequence of their proximity to cultivated trees.

Conclusions Integrating the results of genetic and phenotypic analyses we provide crucial evidence of the presence of a genuine var. *sylvestris* population in the southern Levant, in close geographical proximity to archaeological sites with the earliest evidence of olive exploitation in the ancient world. We supplement the results with recommendations for a conservation program that combines municipal requirements and the urgent need to preserve the largest population of var. *sylvestris* in the southern Levant.

Keywords Conservation, Genetic variation, Morphometry, Oil quality, *Olea europaea* subsp. *europaea* var. *sylvestris*, Spatial structure

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Background

Cultivated olive trees (*Olea europaea* L. subsp. *europaea*) are an integral part of the rural landscape of the Mediterranean Basin. However, as early as the Middle Pleistocene (780 kya BP), records of fossil olive pollen and evidence of charred olive wood and endocarps (pits) appear in the southern Levant, long before olives were cultivated in the region [1, 2]. Thus, it seems that the wild ancestor of cultivated olives, *O. europaea* subsp. *europaea* var. *sylvestris*, has long been an important member of the southern Levant plant community. Moreover, several studies used the presence of fossil olive pollen in sediments as an indicator of past climatic conditions in the Mediterranean [3–5]. In addition, changes in olive pollen abundance in historical periods were used to deduce past human activity [6, 7] and the agricultural landscape of the southern Levant [8, 9]. A palynological study that compared records of fossil olive pollen in 23 sites around the Mediterranean Basin, suggested that the earliest rise in olive pollen in the southern Levant region, around 7,000 y BP, coincided with the onset of olive cultivation [10]. This evidence also coincides with other archaeobotanical and archaeological evidence from around that period [10–13], pointing to the southern Levant region as the primary area of olive domestication. However, the genetic analysis of a large number of samples of wild olive populations and cultivated varieties from around the Mediterranean Basin suggested that olive domestication started in the east Mediterranean, but not specifically in the southern Levant subregion [14, 15]. Diez et al. (2015) [16] suggested an alternative scenario in which olive domestication separately occurred in two independent centers, in the eastern and central parts of the Mediterranean Basin.

Long before the onset of cultivation, olive fruits were collected from wild trees for consumption (table olives) and oil [11, 17, 18]. The natural gene-pool of the wild relatives of olives is considered a source of useful adaptive traits [19–21]. However, the long co-existence of cultivated and wild olive populations, and generations of frequent gene-flow between them, raise questions concerning the existence of genuine var. *sylvestris* in the Mediterranean Basin. Indeed, by utilizing molecular fingerprint markers, many populations of naturally growing olive trees (NGOs) were identified as feral [15, 21–26]. However, NGOs showing contrasting genetic structures from cultivated and feral individuals are commonly considered genuine var. *sylvestris* [15, 23, 26, 27]. Thus, it is reasonable to believe that populations of genuine var. *sylvestris* remained in habitats which restrict gene flow via anemophily or zoochory seed dispersal. Phenotypically, NGOs of var. *sylvestris* possess a bushy form, and a long juvenile stage. In addition, their fruits are remarkably smaller [28], contain a lower percentage of oil [29] than cultivated trees, and possess high variation in fruit

morphology [18]. Feral olives resemble those of wild var. *sylvestris*, thus making the distinction between feral and wild var. *sylvestris* through morphological characteristics difficult.

In the southern Levant, a region that includes Israel, the Palestinian Authority and Jordan [30], the Carmel ridge is considered the southernmost distribution edge of *O. europaea* subsp. *europaea* var. *sylvestris* in the East Mediterranean [31, 32]. Therefore, NGOs south of the Carmel region are considered feral trees [23]. However, feral populations are also common within the original distribution range, and very often NGOs populations include relicts of cultivated trees and their progenies [23]. In this study, we investigated two well-established populations of NGOs: (1) in the Western Galilee (Idmit), where distinct genetic divergence between NGOs and cultivated olives was previously described [23]; and (2) at the southernmost distribution edge of var. *sylvestris*, on kurkar ridges (calcareous cemented quartz sandstone of aeolian origin) along the Carmel coast, near Atlit (Fig. 1). The two investigated populations are located in close geographical proximity to cultivated groves, no more than 2 km apart. However, these populations, located along the Mediterranean coast, are subjected mostly to a west-to-east wind regime [33] that minimizes the likelihood of pollination from cultivated olives growing east, south or north of these sites. We therefore hypothesized that NGOs in Atlit may represent a wild gene-pool of var. *sylvestris* that has been less subjected to genetic erosion by gene flow from cultivated olives. Recently, Tourvas et al. (2023) [26] stressed the immediate need for in situ protection of var. *sylvestris* populations. Here, an integrative eco-genetic approach was designed to understand the spatial genetic structure of two southern Levant NGOs populations, in order to provide recommendations for conservation programs.

Methods

The study area

In Idmit (35°11'41.86"E, 33°04'35.54"N), NGOs growing on cliffs facing the Mediterranean coast were resampled and included in this study as a reference population, representing NGOs of var. *sylvestris* [23]. NGOs in Idmit possess a shrubby juvenile form, a result of continuous grazing by goats and rock hyrax (*Procapra capensis*) (Fig. 1). NGOs in the Idmit were mapped and leaves were sampled for DNA extraction from 17 individuals (Supplementary File #1). In Atlit (34°57'07"E, 32°42'41"N), the largest known NGOs population in the southern Levant, over 1,000 NGOs are distributed in unique coastal habitats in close proximity to the Mediterranean Sea, along three parallel saline Levantine kurkar ridges (Fig. 1). There, a more profound botanical survey was conducted to map and investigate the spatial distribution of NGOs

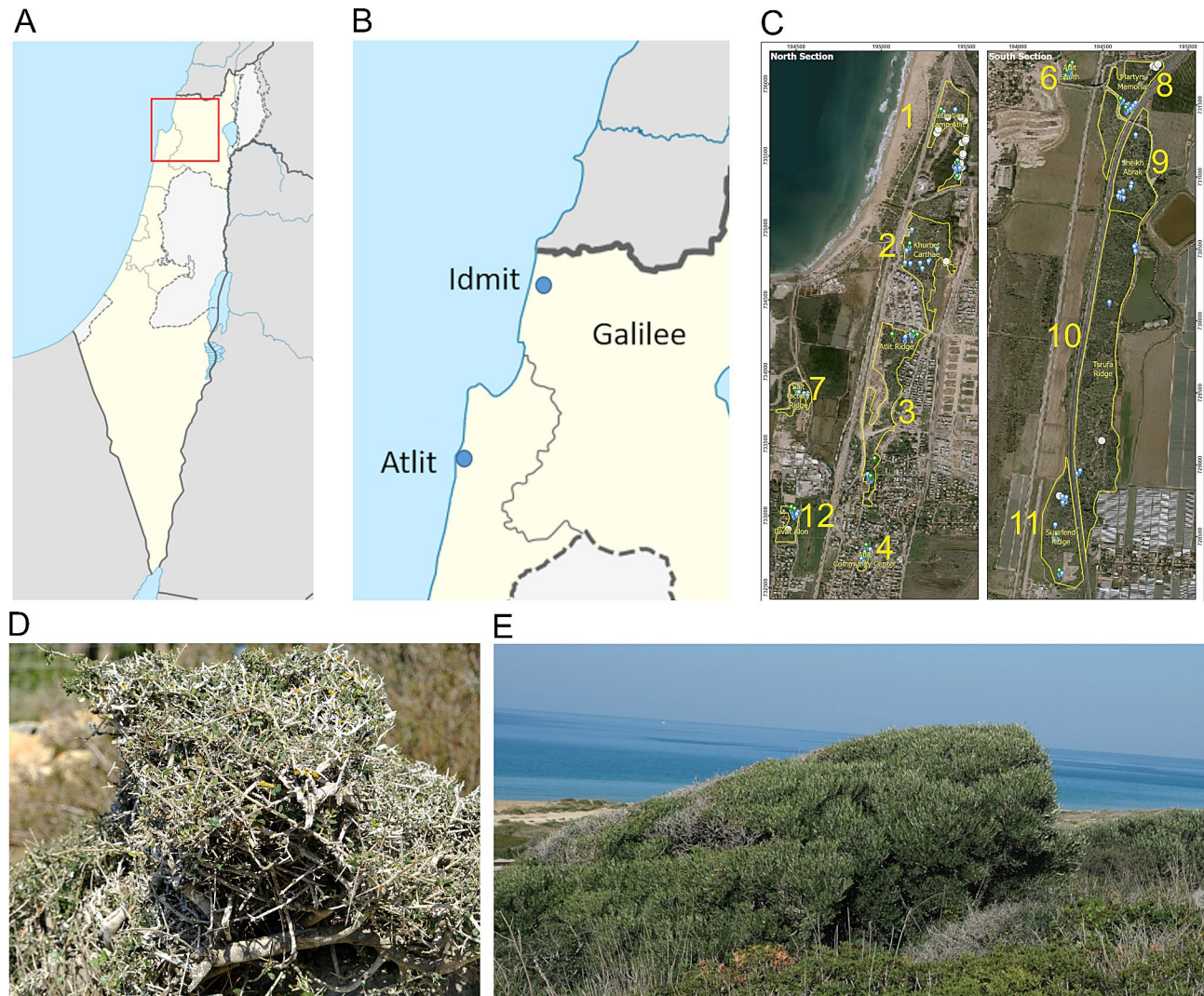


Fig. 1 Mapping naturally growing olives in the Carmel region and Galilee, the presumed distribution range of *O. europaea* subsp. *europaea* var. *sylvestris* in the southern Levant. **(A)** Naturally growing olive trees were sampled in two populations, Idmit, and Atlit. **(B)** In Atlit, naturally growing olives were mapped and sampled in 12 polygons. **(C)** NGOs appearance in Idmit **(D)** and Atlit **(E)** indicate to the seniority of the plants, and thus the long existence of NGOs populations in the region

in a 120-hectare area that was divided into 12 spatially separated polygons of sub-populations (Table 1 and Supplementary File #1). Supposedly, Atlit is at the southwest border of var. *sylvestris* distribution range (Carmel coast) in the East Mediterranean [31].

Naturally growing olive trees at Atlit

NGOs in the 12 polygons in Atlit were mapped, and their phenotypic variation was assessed by characterizing their appearance (well-developed mature vs. young trees) and shape (flagged, bushy and whether it possesses an apparent trunk and suckers - as a proxy of their age). Qualitative estimations included trunk perimeter, canopy size (diameter) and plant height. Fruit load was also evaluated between none (not bearing fruits) and high load. Up to 10 individual NGOs in each polygon (a total of

112 NGOs) were arbitrarily assigned on the resulting maps for sampling canopy leaves for DNA extraction. In November 2022, fruits were harvested from a total of 27 individuals possessing high yields (more than 1 kg from each NGOs) and used for phenotypic assessment of the fruits: average weight and oil content (25 NGOs), fatty acids profiling (20 NGOs) and for morphometric analysis of their pits (23 NGOs); not all NGOs that were sampled for DNA were also sampled for the phenotypic analyses. The number of NGOs in each polygon that were sampled for the genetic and phenotypic characterization is listed in Table 1, and their location is marked on the maps presented in Supplementary File #1.

Table 1 The number of NGOs mapped in the 12 polygons at Atlit, and those sampled for genetic and phenotypic analyses (cf. maps in supplementary file #1)

Polygon	#NGOs	Genetic analysis	Phenotypic analysis
1	311	10	3
2	102	10	1
3	100	10	6
4	31	10	3
5	42	10	3
6	11	8	3
7	67	7	1
8	100	10	2
9	76	10	1
10	117	10	0
11	39	8	2
12	59	9	2
Total	1055	112	27

DNA extraction and genetic analysis

DNA was extracted using the CTAB method. After a quality check on agarose gel, DNA was quantified using a NanoDrop spectrophotometer (DeNovix DS-11, NC, USA), diluted and PCR reactions were performed using 14 SSR loci (simple sequence repeats) (Supplementary Table S1) that were previously tested and showed polymorphic, clear, and scorable profiles [23, 34]. The analysis included DNA samples of two multi locus analysis (MLL) genetic groups, representing two traditional local cultivars (TCs) that exist among local old living olive trees, MLL1 and MLL7 [34]. DNA was extracted from leaves that were collected from trees of each of MLL1 and MLL7 groups (13 and 11 samples, respectively), growing in a live germplasm collection at the Gilat Research Center (34°39'57.3"E, 31°20'08.3"N) (Supplementary Fig. S1), which includes olives developed from propagated material of local TCs [34].

PCR products were separated on an ABI automated sequencer (Applied Biosystems, 3730xl DNA Analyzer 96-Capillary Array (Thermo Fisher, USA); Center of Genomic Technologies, the Hebrew University of Jerusalem) as a multiplex of several loci labeled with three different fluorescent dyes (FAM-6, TAMARA and HEX, Applied Biosystems; see Supplementary Table S1 for loci used in each Multiplex pool), using the size standard GeneScan 500 LIZ. Electropherograms were then scored manually using Genmarker 1.75 (SoftGenetics, State College, Pennsylvania, USA).

Genetic analysis included the analysis of multi-locus genotypes (MLGs) and grouping those into MLLs using Genotype 1.2 [34]. One sample of each MLL was then used to calculate genetic diversity values (allele number, expected, observed and unbiased heterozygosity) using GenAlEx v6.5 [35]. F_{ST} genetic distances were calculated using GenAlEx v6.5 to infer the genetic distances

between populations and local cultivars. In addition, gene flow was assessed by calculating G_{ST} values by GenAlEx v6.5 [26]. Principal coordinate analysis (PCoA) was performed on the standardized covariance matrix of genetic distances calculated according to Smouse & Peakall (1999) [36] using GenAlEx. Finally, Structure V.2.3.4 [37] was used for Bayesian clustering with the admixture model, to assign each MLG from each of the populations to K clusters. Ten independent runs for given Ks (2 to 9) were performed with a burning length of 10,000, followed by 20,000 repetitions [38]. The log likelihoods for a given K were used to choose the best given K based on an ad hoc quantity of ΔK [39]. Visualization of the results was made with *distruct* ver. 1.1 (<https://rosenberglab.stanford.edu/distruct.html>).

Fruit phenotype assessment

One hundred fruits, a subsample randomly selected from the above-mentioned harvested fruits from each of the 25 individual NGOs harvested in Atlit, were used to determine the average weight of a single fruit. Following Naor et al. (2013) [40], the oil content was determined on the fruit paste using the OliveScann near infrared (Foss, Denmark). A laboratory oil mill (Abencor, MC2 Ingenieria y Sistemas, Spain) was used to extract virgin olive oil from 1 kg of fruit from 20 NGOs individuals (Atlit) and 11 trees of MLL1 TC, used as a reference of a local cultivar. Oil was not extracted from fruits of MLL7 as they ripe early in the season and exhibit a different maturity index than those of NGOs and MLL1. Fatty acid (FA) composition in the oil was determined after cold methylation using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), coupled to a 5799 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) [41]. FAs were identified according to standard compounds and their relative composition in the oil was determined as the percentage of total FA. The resulting data set was used for PCA (principal component analysis) and statistical analysis using JMP 16 (SAS Institute, Inc.).

Morphometric analysis of olive pits

Pits were recovered from at least 20 randomly selected fruits from each of the 23 NGOs (Atlit), and fruits of the MLL1 and MLL7 TCs, as references of local cultivars. Olive pits were recovered by manually scrubbing the pulp (mesocarp) with a plastic abrasive sponge, and the pits were then washed in a 2% hypochlorite solution to digest the remains of the pulp. Each olive pit was photographed on a blue background with a mm scale with a Canon EOS 200D camera equipped with EFS 18–55 mm lens. Separate photos were taken vertically for a dorsal and lateral view of each pit. Several cleaning steps were conducted using the GIMP image editing software as

required (version 2.4.1.3, <https://www.gimp.org/release-notes/gimp-2.4.html>), and a dedicated MATLAB code was used for cropping, filtering and rescaling the images. The images were then filtered and sharpened using a two-dimensional median filter and standard unsharp masking method, by applying a Gaussian lowpass filter. The filtered and sharpened images were then filtered using a 2-dimensional convolution filter with an adjustable kernel window size; binarization, connectivity and erosion factors, that were manually adjusted to best trace the silhouette of each pit. The boundaries of the pits were traced using the Canny approach for edge detection. The analyzed features included area, circularity, convex area, eccentricity, equivalent diameter, major axis length, maximum ferret diameter, minor axis length, minimum ferret diameter, orthogonal diameter, perimeter, solidity, roundness, aspect ratio, form factor and compactness for the dorsal and lateral view of each pit. PCA was performed on the resulting morphometric dataset using R, after scaling each feature using Z-score normalization by column.

Results

Naturally growing olive trees in Atlit

A total of 1,055 NGOs were mapped in Atlit (Table 1 and see maps in Supplementary file #1). These represent only well-established NGOs, whereas young saplings were excluded. The majority of the mapped individuals (94%) were well-developed senior trees that included trees with apparent trunks and substantial canopies but maintaining a bush form (Supplementary Fig. S2 and File #2). Fruit load varied among the well-developed olive trees, ranging from trees bearing no fruit at all to high fruit

load (Supplementary Fig. S2 and Files #1 and #2). Fruits were collected from NGOs from the latter group for phenotypic assessment (below). A considerable number of NGOs in Atlit had only juvenile leaves or both juvenile and mature leaves (104 and 475 trees, respectively). Young NGOs, $n=59$, showed an early juvenile stage, thus additional data were not collected (Supplementary File #2). Field observations indicate that of the 444 NGOs mapped in a west aspect facing the Mediterranean Sea, 85 possess a Krummholz (flag) formation (Supplementary Fig. S2 and File #2, cf. Figure 1E). Those were found in polygons 1, 2, 7, 8, 10 and 11, on the kurkar ridge facing the Mediterranean Sea.

Genetic analysis

The analysis of 14 SSR loci resulted in allele numbers ranging from 3 to 29 (Supplementary Table S1). The number of alleles in the two studied populations was twice as high as their number in the local MLL1 and MLL7 TC (Table 2). Noticeable differences between the NGOs and TCs also appeared in their heterozygosity values, with higher values of unbiased heterozygosity (uHe) than observed heterozygosity (Ho) in NGOs and the opposite in the two MLL groups. However, NGOs sampled in polygon #5 in Atlit, within the residential area, showed substantially higher Ho and uHe values (0.730 and 0.748, respectively) than other NGOs (Table 2). PCoA analysis provided further evidence of higher genetic diversity among the NGOs in comparison with the TCs, which were grouped closely together in two separate clusters (Fig. 2).

The largest G_{ST} values were detected between MLL1 and MLL7 (0.174), as expected from vegetatively propagated cultivars (Fig. 3). A large G_{ST} value was found between the NGOs in Idmit to both MLL1 and MLL7 (0.136 and 0.098, respectively). Calculating G_{ST} values separately for each polygon at Atlit showed the largest G_{ST} values between NGOs in polygon #7 and MLL1 and MLL7 (0.126 and 0.116, respectively). Relatively small G_{ST} values between NGOs and MLL7 were found in polygon #9 (0.053) and between NGOs and MLL1 in polygon #2 (0.069) (Fig. 3).

In general, pairwise genetic distances values between populations of NGOs and MLL7 were lower than between NGOs and MLL1, as reflected in the PCoA (Figs. 2 and 3). Relatively low genetic pairwise distances were found between NGOs of the two populations and within the subpopulations at Atlit, reaching high pairwise distances between the NGOs in Idmit to those in polygon #3 in Atlit ($F_{ST}=0.102$). Relatively higher F_{ST} values were found between the NGOs of Atlit and Idmit and the two groups of cultivated trees. NGOs in polygon #9 in Atlit showed the lowest pairwise distances to MLL7 (0.073). Similarly, the lowest F_{ST} value was found between NGOs

Table 2 Genetic diversity among naturally growing olives in Idmit and Atlit, and the main local MLL1 and MLL7 traditional cultivars: number of different alleles (na), and observed (Ho) and unbiased expected (uHe) heterozygosity

Population	Polygon	N	Na	Ho	uHe
Idmit		17	5.714	0.591	0.717
Atlit	1	10	5.429	0.507	0.695
Atlit	2	10	5.643	0.541	0.709
Atlit	3	10	5.071	0.435	0.649
Atlit	4	10	5.571	0.562	0.706
Atlit	5	10	6.214	0.730	0.748
Atlit	6	8	5.143	0.541	0.702
Atlit	7	7	4.429	0.565	0.701
Atlit	8	10	6.000	0.609	0.705
Atlit	9	10	6.286	0.654	0.775
Atlit	10	10	6.429	0.652	0.749
Atlit	11	8	4.714	0.579	0.726
Atlit	12	9	5.214	0.495	0.723
MLL1		13	2.357	0.819	0.469
MLL7		11	2.786	0.968	0.565

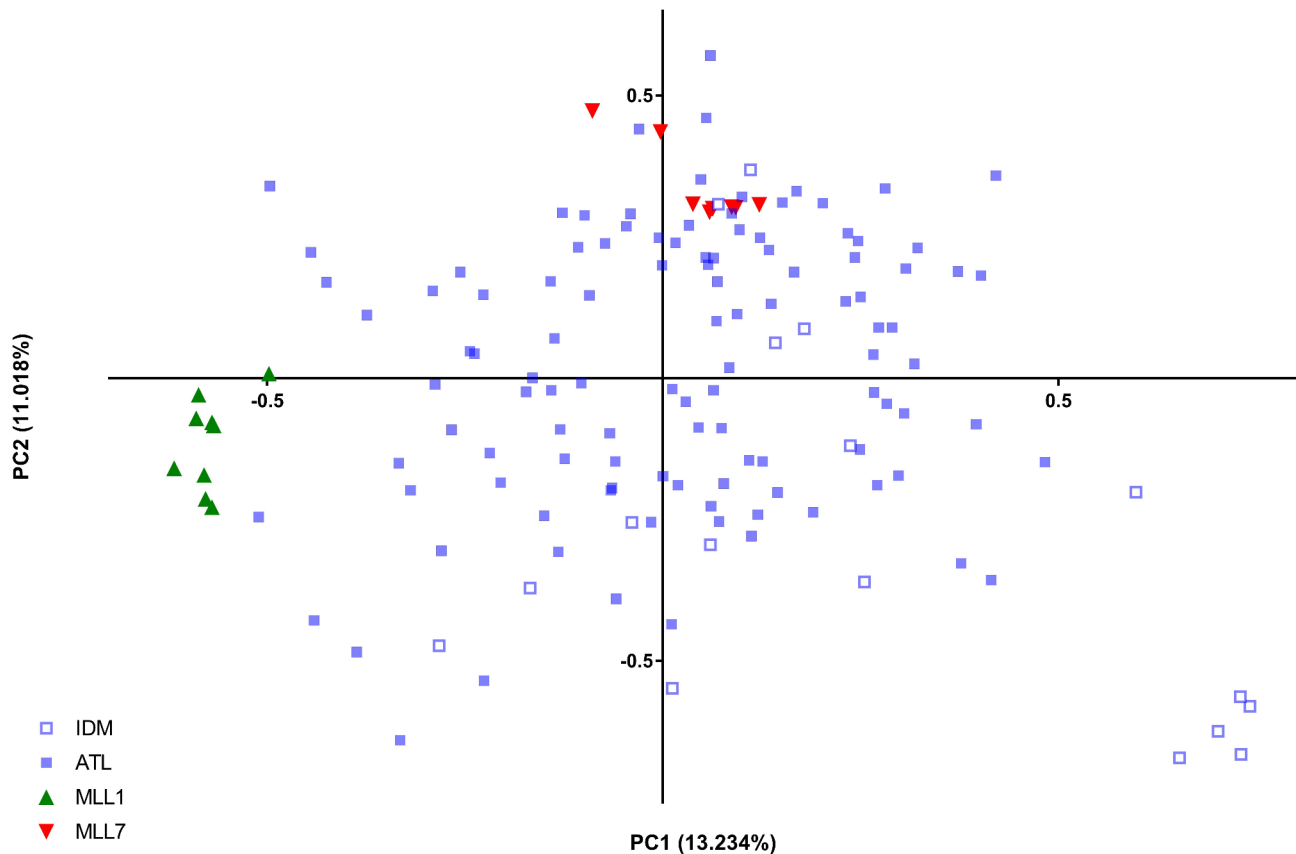


Fig. 2 Principal coordinate analysis (PCoA) of the two studied NGOs populations (Idmit and Atlit), MLL1 and MLL7 represent the most common southern Levant traditional cultivars

in polygon #2 and MLL1 (0.089) (Fig. 3). Relatively low genetic distances were also found between NGOs in polygon #4 and #5 to MLL7 (Fig. 3).

Assigning the NGOs (and samples of the two TCs as a reference group) to MLLs indicated that the majority of NGOs in Idmit and Atlit were assigned to a single occurring MLL, genetically different from any other sample included in the analysis (Table 3). The spatial distribution of MLLs in the mapped populations is presented in Supplementary File #1. However, in Idmit, 10 out of the 17 sampled NGOs were assigned to three site-specific MLLs (Table 3 and maps in Supplementary File #1), providing further support to a high frequency of clonal reproduction at this site [23], as was similarly shown in NGOs at Atlit (polygon #9, Supplementary File #1). Additionally, no evidence for local olive TCs was found in either Atlit or Idmit (Table 3), unlike in previous investigations of several other NGOs populations in the Carmel Ridge and Galilee [23].

Based on genetic distances, applying the Bayesian admixture model for $K=2$ to 9, showed a clear peak of ΔK at $K=8$, the optimal number of subgroups (Supplementary Fig. S3). The dominance of the two TC clusters was evident in all K (Fig. 4, cf. Table 2). At Idmit, the genetic

structure provided further evidence of clonal vegetative propagation of several NGOs. The maps in Supplementary File #1 show the close proximity of NGOs sharing the same MLL in Idmit, and similarly in Atlit (polygon #9). Nevertheless, at K8, NGOs of both Idmit and Atlit showed admixture genetic structure (Fig. 4).

Fruits characteristics

The average fruit weight of NGOs at Atlit was substantially (2.8 times) lower than those of MLL1 (0.95 ± 0.36 and 2.64 ± 0.15 g, respectively; t -test, $P < 0.001$), as was the case for the relative oil content in the fruit (7.4 ± 0.52 and $16.32 \pm 1.05\%$, respectively; t -test, $P < 0.01$) (Fig. 5). However, no difference in relative water content was found between NGOs and MLL1 olive pastes ($\sim 58.0\%$).

PCA separated the NGOs from cultivated olives on the basis of 12 detected FAs in the oil (Fig. 6 and Supplementary Table S2). In addition, NGOs fruit oil extracts from Atlit showed substantially larger variation in the relative content of FAs in comparison to the cultivated MLL1 oil extracts (Fig. 6). The first component of the PCA was mostly dominated by oleic, linoleic, gondoic and palmitic FAs and the second by behenic FA. Significant differences (t -test, $P < 0.001$) were found between the two groups of

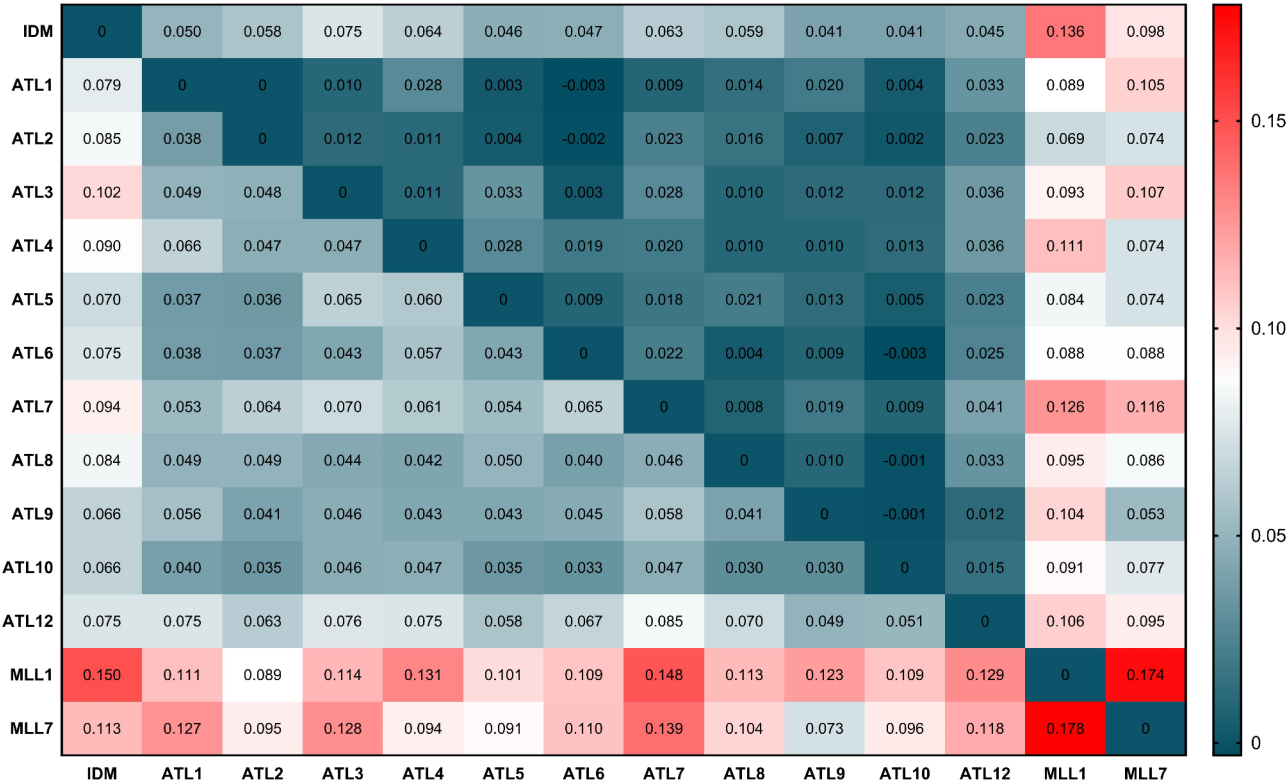


Fig. 3 Pairwise F_{ST} and G_{ST} values between NGOs of two investigate populations, Idmit (IDM) and Atlit (ATL), and MLL1 and MLL7 traditional cultivars. G_{ST} and F_{ST} values are presented above and below the diagonal, respectively

Table 3 Number of naturally growing and cultivated olives assigned to different multi-locus lineages (MLL). The number of trees assigned to each MLL1, MLL7, single occurring (SO) MLL, and the total number of MLLs found in each population are given. MLL1 and MLL7 represent the most common MLLs found in old cultivated trees [34]

	Idmit	Atlit	MLL1	MLL7
N	17	112		
MLL1	0	0	13	
MLL7	0	0		11
SO MLL	7	111		
Total MLL	11	112	1	1

olive in the relative content of the abovementioned FAs, showing significantly higher relative oleic and gondoic FA content in the NGOs oil than in MLL1 (Fig. 7).

Morphometric analysis of olive pits

The PCA (Fig. 8), after normalization of the dataset, showed that most of the variation was dominated by three main pit features: (a) size (e.g., length, diameter, area); (b) shape (e.g., circularity, compactness, roundness and form factor); and (c) specific shape features, i.e., eccentricity and aspect-ratio (Supplementary Fig. S4). The PCA demonstrated clear differences between pits of the Atlit NGOs and the two TCs (Fig. 8). In addition, pits

of NGOs showed considerably higher variation in size and shape (Figs. 8 and 9).

Discussion
Genetic evidence supports the existence of *O. europaea* subsp *europaea* var *sylvestris* in the southern Levant
The results of the genetic analysis (Figs. 2, 3 and 4; Tables 2 and 3) indicate that the NGOs in Atlit represent the largest known population of *O. europaea* subsp. *europaea* var. *sylvestris* in the southern Levant. In addition, the results further support the existence of the wild olive form in Idmit [23]. A similar SSR analysis that showed strong genetic differentiation between NGO populations and olive cultivars recently suggested the existence of populations of var. *sylvestris* in Greece [26]. In addition, by using a target gene approach in analyzing 27 NGO populations, Zunino et al. (2024) [42] proved the existence of a few populations of genuine var. *sylvestris* in Morocco, France and Spain. However, the majority of the investigated populations, both in Greece and in the western parts of the Mediterranean Basin, showed admixed genetic structures and were identified as feral, regardless of their distance from cultivated groves [26, 42]. Palynological studies show that olive pollen can reach distances far away from olive groves, as in the case of olive pollen records documented in sediment layers near the Dead

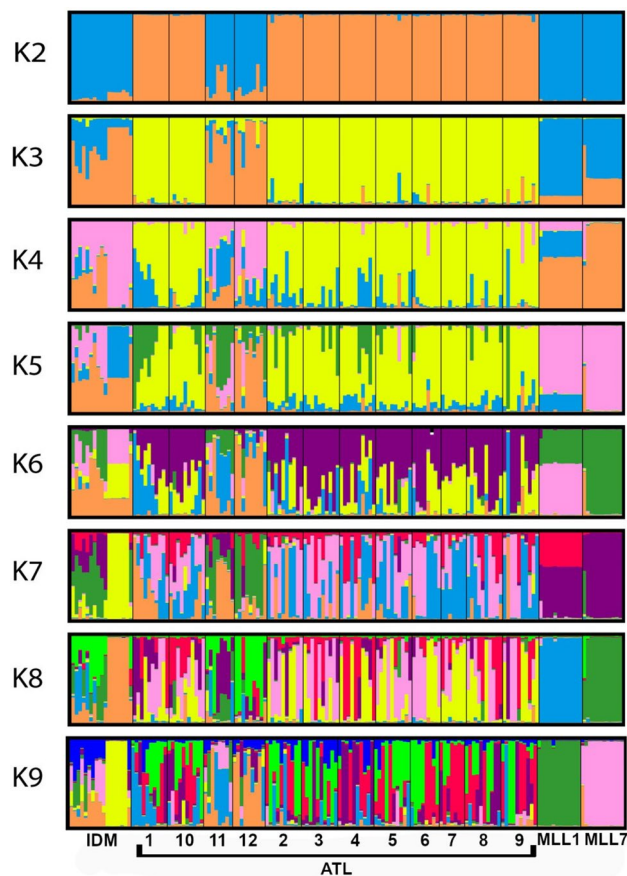


Fig. 4 Inferred genetic structure of naturally growing olives and of the two main local traditional cultivars MLL1 and MLL7. Bayesian clustering with the admixture model implemented in Structure was used to assign individual MLGs to genetic clusters ($K=8$, Supplementary Fig. S3). Individual MLGs within each group are represented by vertical bars and genetic groups are shown in different colors

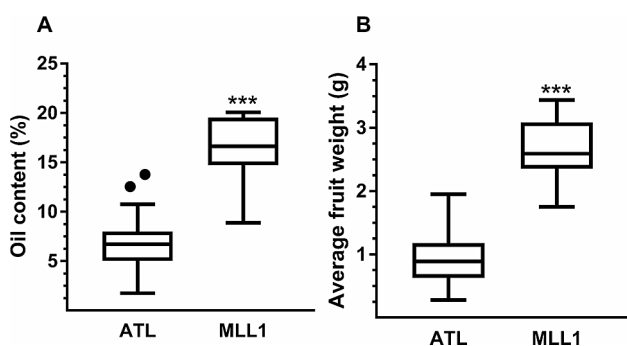


Fig. 5 Box-and-Whisker diagrams showing the means with upper and lower variability of the oil content in the fruit (A) and average fruit weight (B) of naturally growing olives in Atlit and MLL1. Asterisks represent significant differences at $P < 0.001$ level

Sea, even before the onset of olive cultivation [43]. Moreover, modeling olive pollen intensity shows that in elevations below and above 300 m a.s.l., olive trees can be pollinated by pollen from as far as 59 and 27 km, respectively [44]. Thus, considering that olive is an allogamous

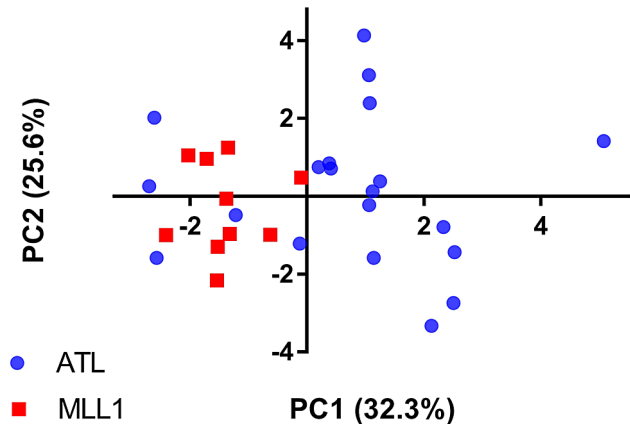


Fig. 6 Results of the principal component analysis (PCA) of chemical compounds in the oil of the two groups of olive, cultivated MLL1 and naturally growing olives in Atlit (red and blue symbols, respectively) (see Table S2). Percent of variance explained by each axis is indicated in the axis titles

species that relies on anemophily, and millennia of possible gene-flow between cultivated olives and NGOs, it was surprising to find the presence of any wild olive in the southern Levant. However, considering that in this region, the Idmit and Atlit sites are at the edge of the Mediterranean coast (Fig. 1), where NGOs are subjected to a specific wind regime that minimizes pollination probability by cultivated trees, our results support the hypothesis that specific insulated niches provide enough protection to maintain genuine populations of var. *sylvestris* [26].

Signs of inbreeding ($uHo < He$, Table 2) suggested a high degree of isolation of the two populations of Atlit and Idmit [23]. However, the G_{ST} and F_{ST} values (Fig. 3), and the admixed population genetic structure (Fig. 4), suggests some influence of cultivated trees at Atlit on the genetic structure of the NGOs in polygons #2 and #9 and to a lesser extent in polygons #4 and #5. A recent land-use survey conducted in the Carmel region indicated that cultivated olives and olive groves are among the main components of the Carmel landscape, encompassing up to 22% of the land along the Carmel coastal plain and the Carmel mountain ridge [45]. Moreover, polygon #4 and #5 are situated within the Atlit residential area, where cultivated trees are present in private and public gardening. In addition, the NGOs in polygons #2 and #9 grow around two old settlements: the ancient medieval fortified 'Casel Destreiz' (or 'le Destroit') in polygon #2 [46], and an Armenian village established in 1920 and finally deserted in 1981 in polygon #9 [47]. It can thus be assumed that NGOs in these four polygons show an intermediate genetic structure in the transition between the wild var. *sylvestris* and cultivated olives, and exemplify the fate of the population if immediate action is not taken.

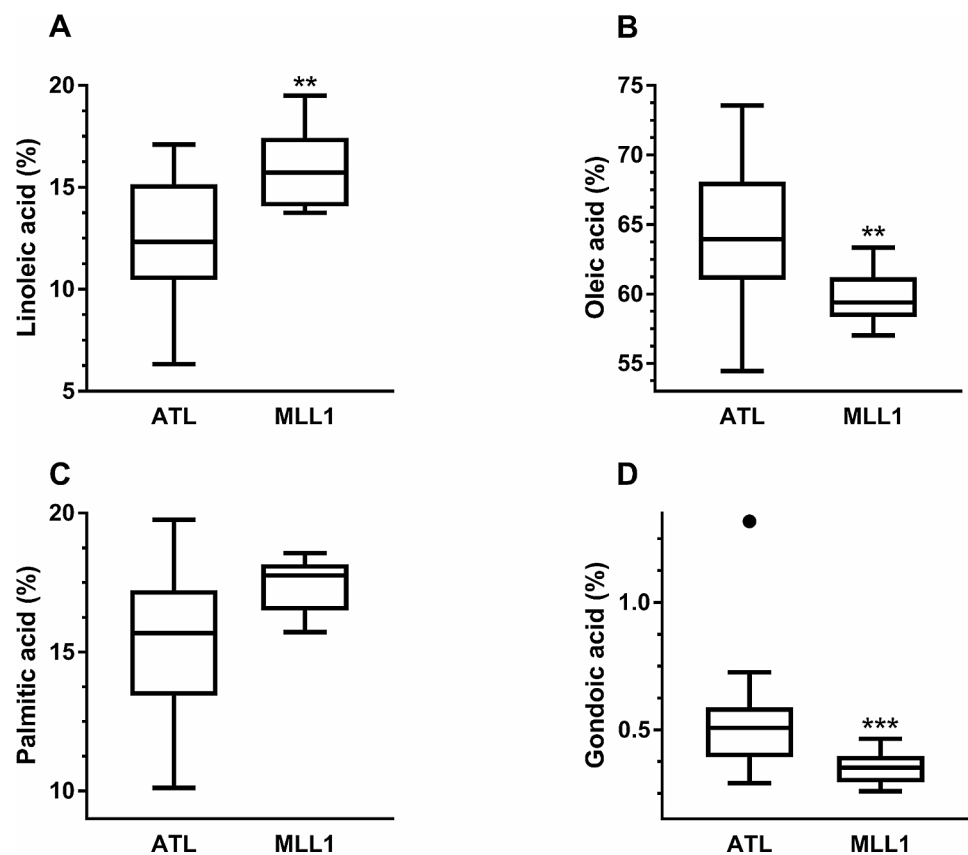


Fig. 7 Box-and-Whisker diagrams showing the median with upper and lower variability in the relative content of linoleic (A), oleic (B), palmitic (C) and gondoic (D). FAs of naturally growing olives in Atlit and the MLL1 cultivar. Asterisks represent significance at $P < 0.01$ (**) and $P < 0.001$ (***) levels

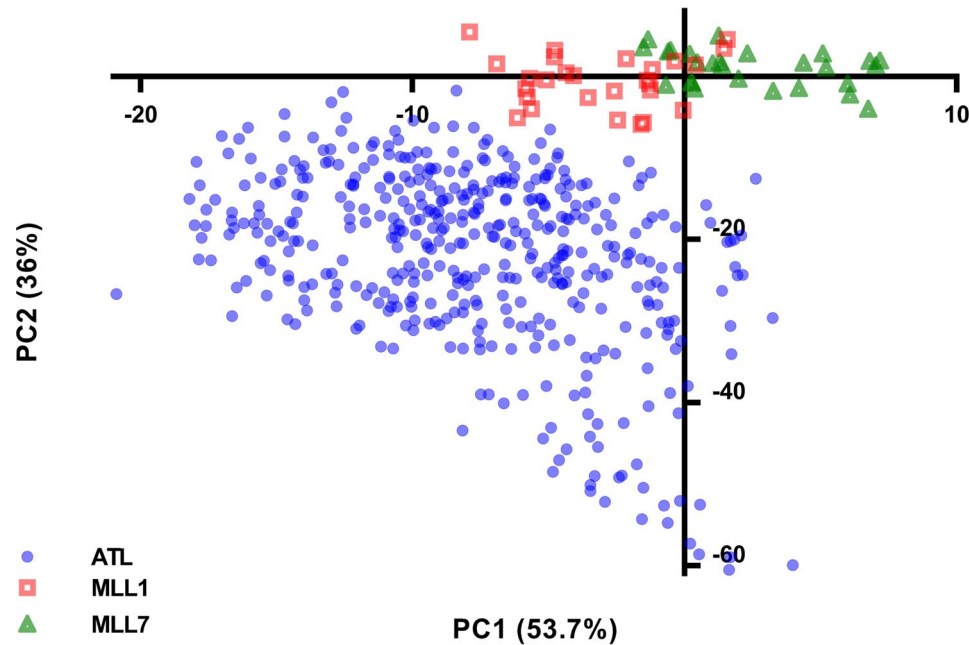


Fig. 8 Results of the principal component analysis (PCA) of morphometric characteristics of NGOs olive pits NGOs from Atlit and those of the cultivated traditional cultivar reference groups: MLL1 and MLL7. Percent of variance explained by each axis in the PCA is indicated in the axis titles

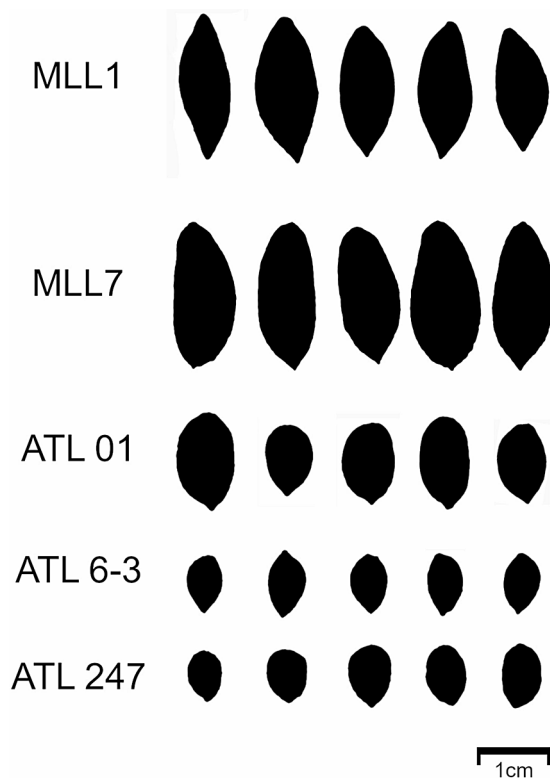


Fig. 9 Representative pits of naturally growing trees from Atlit in comparison to those of the cultivated reference varieties

Phenotypic differences between *Olea europaea* subsp. *europaea* var. *sylvestris* and cultivated olivest

The NGOs in Atlit enabled further investigation of the phenotypic traits of var. *sylvestris*. The NGOs in Atlit varied in size, shape, appearance and formation (Supplementary File #2 and Fig. S2), all indicating a well-established population. Fruits of NGOs were considerably smaller and contained lower oil content compared to cultivated trees (Fig. 5), traits that are typical to var. *sylvestris* [28, 48]. In addition, PCA showed a clear divergence between MLL1 and NGOs in the relative FAs content (Fig. 6), although the oil of both groups was dominated by oleic, palmitic and linoleic FAs (Supplementary Table S2). Considering that the fruits of NGOs and MLL1 were collected in two distinctly different environments, the results may reflect environmental factors on olive quantity and quality, particularly the effect of irrigation [49]. However, the relatively low oil content measured in fruits of MLL1 (ca. 16%) and the relative content of FAs in the oil are in the range reported for MLL1 across different environmental conditions [50].

Olive pits, unlike fruits, exhibit a conservative morphology that is not influenced by environmental factors, thus allowing the investigation of genetic variations [18]. Results of the PCA summarizing the analysis of 16 morphometric characteristics of NGOs and reference

cultivars (Fig. 8 and Supplementary Fig. S4), indicated that image processing is useful in creating morphometric passport data for cultivar identification [51]. PCA summarizing the total analyzed traits showed a clear divergence between the NGOs and cultivated varieties (Figs. 8 and 9). Pits of NGOs possessed large morphometric variation and were considerably smaller than those of cultivated varieties. However, feral olives also exhibit large variations in morphological traits and distinct shapes and sizes of fruits and pits that are different from cultivated olives [52, 53]. Nevertheless, altogether, the genetic analysis, in combination with phenotypic and morphometric analyses, provides support to the identification of a large NGOs population of *O. europaea* subsp. *europaea* var. *sylvestris* in Atlit.

Conservation recommendations

In situ genetic reserves ensure the protection of populations of plant genetic resources in their natural habitats. Protected genetic reservoirs allow evolutionary processes to continue and thus are considered a complementary approach to *ex situ* conservation (i.e., in live germplasms, botanical gardens and gene banks). Increasing concern about the loss of valuable genetic diversity have accelerated local conservation programs in Israel, with special emphasis on *ex situ* seed conservation of crop wild relatives and endangered species [54, 55]. Local plant genetic resources in Israel are protected in 230 terrestrial nature reserves, but only the Amiad reserve was declared as an in situ genetic reserve of emmer wheat (*Triticum turgidum* var. *dicoccoides*) and several other cereal wild relatives [56–58]. The NGOs in Idmit and those growing in polygons #2 and #10 in Atlit are found within natural reserves (Fig. 1 and Supplementary File #1). However, G_{ST} values, genetic distances (F_{ST}) and the admix genetic structure in several polygons (#2, 4, 5 and #9) at Atlit possibly reflect the influence of nearby cultivated trees, remnants of groves in old settlements or those planted in public and private gardens. Nevertheless, the majority of polygons at Atlit, and polygon #7 specifically, remained a sanctuary for *O. europaea* subsp. *europaea* var. *sylvestris*. In Idmit, grazing by rock hyrax and goats shape the appearance of olives (Fig. 1) and also hinders flowering, which so far may be responsible for the limited possible gene-flow from cultivated olive trees planted in a nearby recreational park (as reflected in the G_{ST} values, Fig. 3).

Development and urbanization processes necessary to meet the demands of an ever-increasing human population in the southern Levant, and specifically in Israel, rapidly increase the risk of genetic resource loss (<https://chm.cbd.int/database/record?documentID=252812>, in Hebrew). In the town of Atlit itself, an on-going municipal development program threatens the existence of the NGOs population, both by clearing areas for housing and

planting cultivated trees in public and private gardens. Our results suggest the inclusion of specific NGOs in the municipal programs to ensure their protection. In addition, in order to preserve the genetic integrity of NGOs in Idmit and Atlit in the future, park and municipal gardening should avoid planting olive trees and transplant those already planted in the Atlit and Idmit recreational parks elsewhere.

Conclusion

More than 30 years of monitoring the genetic diversity of emmer wheat populations in the Amiad genetic reserve (eastern Galilee) has demonstrated that the establishment of a small, protected site is enough for effective in situ conservation [58]. Thus, the protection of NGOs within the nature reserves in Atlit and Idmit, with appropriate management, should ensure the maintenance of the last two remaining populations of *O. europaea* subsp. *europaea* var. *sylvestris* in the southern Levant. This unique germplasm possesses valuable potential for academic and agronomical studies, especially in global climate change hotspot [59, 60]. Finally, the close proximity of these populations to the archaeological sites with the earliest evidence of olive exploitation in the ancient world [11, 12] emphasizes the importance of designating these populations as heritage resources and conducting the necessary actions to ensure their protection and preservation for future generations.

Abbreviations

BP	Before Present
FA	Fatty Acid
MLG	Multi-Locus Genotypes
MLL	Multi Locus Analysis
NGO	Naturally Growing Olives
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
SSR	Simple Sequence Repeats
TC	Traditional Cultivar

Supplementary Information

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Supplementary Material 1
Supplementary Material 2
Supplementary Material 3

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Author contributions

OB, AD, EG and AP conceived the study. AP and DLR conducted the botanical survey. Sampling leaves and fruits for genetic and phenotypic analyses was performed by EBD, TC and DLR. The molecular work was performed by EBD and TC, and they conducted the data analysis together with BH. EBD and YBD developed and conducted the morphometric analysis, and ZT performed the olive oil analysis. EBD and OB wrote the manuscript with the contribution of all authors.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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