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Article

Wild Grapevine (*Vitis vinifera* L. subsp. *sylvestris* (C.C. Gmelin) Hegi)—Novel Species to the Israeli Flora

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Abstract: The wild grapevine, *Vitis vinifera* subsp. *sylvestris* grows naturally throughout the northern hemisphere, including the Mediterranean region. Wild grapevines were also observed sporadically across the southern Levant and have been considered a non-native feral plant. Nevertheless, no formal characterization was conducted for wild grapevines in this region; thus, its taxonomical assignment remains elusive. Previously we have shown that the wild grapevine populations growing in northern Israel are genetically separated from the feral domesticated forms. The aim of this work was to comprehensively describe the morphological, anatomical, and ecological traits of wild grapevines naturally thriving in two distinct habitats in Israel. The dioicous nature of the wild grapevine, as well as their flower and pollen morphology, their characteristic sylvestris fruit and seed morphology, in addition to the occurrence of natural germination of seeds in close vicinity to the mother plant, all lead to our conclusion that these plants belong to *Vitis vinifera* subsp. S*ylvestris*, and should be included in the Flora Palaestina. These findings, combined with the recently-published genetic evidence for these populations significantly advance our understanding of the species' ecology and persistence under climate change.

Keywords: Israeli flora; morphological characterization; sylvestris; *Vitis*; *Vitis vinifera* subsp. *sylvestris*; wild grapevine species persistence

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1. Introduction

The *Vitaceae* family includes mostly shrubs and woody lianas that climb using leaf-opposed tendrils. Most grape cultivars belong to the *Vitis* genus, consisting of 83 subordinate taxa [1] that exist primarily in the Northern Hemisphere, including North America and East Asia. The Eurasian species of common grapevine, *Vitis vinifera* subsp. *sylvestris* (C.C. Gmelin) Hegi) [2] (hereafter Sylvestris) is the best-known as it is the ancestor of most cultivated grapes grown today [3–5]. The cultivated form *V. vinifera* subsp. *sativa* (hereafter Sativa) is one of the most notable perennial crops, which is cultivated across 7.3 million hectares worldwide [6]. The distinction between these two subspecies relies mainly on the differences in the morphology of the reproductive organs: while the wild grapevine is dioecious, Sativa is a hermaphrodite [5,7,8].

Wild grapevine (Sylvestris) was abundantly growing at natural habitats in Europe until the mid-19th century, when the invasion of pests and pathogens from North America, including phylloxera, powdery and downy mildews, caused a decrease in their populations [8]. Later, the accelerating urbanization processes and extensive anthropogenic land use dramatically damaged the natural habitats of wild grapevine (Sylvestris) populations, reducing its distribution range and endangering the species persistence [9,10]. While Sylvestris populations were shrinking, cultivated grapevine

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(Sativa) was flourishing throughout Europe and the Eastern Mediterranean region, where by the end of the 19th century, according to Post's seminal work, it was "cultivated everywhere in numerous varieties, but nowhere strictly spontaneous" [11].

The Southern Levant was considered a region beyond the distribution range of wild grapevine; thus, early studies of Israeli vegetation during the 20th century considered all grapevine plants as Sativa, i.e., feral populations. Indeed, feral grapevines may emerge due to vineyards being abandoned or neglected, wherein the previously cultivated grapevines are allowed to proliferate without human intervention. Over a period of time, seeds originating from these plants may germinate and be mistakenly observed as wild grapevines, adopting similar climbing growth habits. Therefore, the species was not included in the local wild flora records until 2004 [12]. The first indication of wild grapevine in the Southern Levant region was in 1994, based on surveys in the Upper Galilee region, along the banks of the Jordan River [13,14]. Nevertheless, the available records of these surveys lack the necessary description and exact location of the observed plants.

Over the years, more evidence for indigenous Southern Levantine Sylvestris has accumulated. Fossil pips, pollen, and wood of wild grapevines were discovered in the region from Lower Paleolithic Gesher Benot Ya'aqov (780,000 BP) through Upper Palaeolithic Ohalo II (23,000 BP) sites [15–19]. These archaeological sites are located around the area of the Hula lake, the Sea of Galilee, and the Jordan River, in high geographic proximity to the populations observed in the 1995 survey.

Recently, a dedicated comprehensive survey of grapevines in Israel discovered new populations of hermaphrodite and dioecious plants [20]. Their genetic analysis with SSR markers and morphological characterization revealed that the hermaphrodite accessions are clearly separated from the dioecious groups, having unequivocal wild grapevine characteristics (leaf, bunch, and berry shape, etc.). Moreover, dioecious wild grapevine (Sylvestris) accessions were further split between two distinct subgroups in accordance with ecogeographic divergence. The dioecious populations, occurring primarily along the main streams in the Upper Galilee region and around the Sea of Galilee, mark the southern edge of the distribution range of wild grapevine [21].

Deeper analyses of these accessions using whole genome sequencing data further supported the previous observations that wild grapevine has grown naturally in the Southern Levant for Millenia [22]. In fact, these wild grapevine accessions were identified as progenitors of domesticated indigenous varieties from the Levant with genetic contributions to some of the European varieties [22,23]. These conclusions were further supported in a recent comprehensive genomic study of more than 3,500 accessions which provided an unequivocal evidence for the contribution of Southern Levantine Sylvestris populations to domesticated grapevine around the world [24]. The Sylvestris populations from Israel (E1) were identified as the genetic source of the table-grape group (CG1) [24]. This group later genetically contributed to the most known wine grape varieties used in modern times worldwide.

Despite the number of studies and evidence for the thriving of wild grapevine populations in Israel, an absence of a conclusive report for the species is a noticeable gap for the understanding of the species evolution, geography, ecology, and domestication. Here, we provide a systematic and comprehensive morpho-anatomical characterization of the wild population that spread between two different sites in Israel, including a thorough characterization of male and female flowers, fruit and seed morphology, the distribution dynamics of male and female individuals in each group, and the natural regeneration by spontaneous germination. The results clearly affirm that the wild grapevine populations inspected are truly assigned to the protologue and type specimens of *Vitis vinifera* subsp. *sylvestris*.

2. Materials and Methods

Plant material and research area: Sampling was carried out during the spring (May 2022) in Northern Israel, where stable populations of wild grapevines denoted here as Sylvestris was previously observed [21]. Thirty-two wild grapevine accessions were collected from the Beit Tsaida site located next to the Sea of Galilee (32°53'09.2 "N 35°38'34.6" E), and fourteen accessions were collected from the Banias River site in the Upper Galilee (33°12'12.3 "N 35°38'27.6" E), from an area of

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about 8000 m^2 in each site (Figure 1). The geographical positions of the wild grapevine accessions were georeferenced using the free application Google Maps (Google, Inc.). For each accession, branches with young and mature leaves, as well as inflorescences, were collected. A subsample of each accession was dried and prepared for Herbaria deposition, and the remaining parts were fixed and stored in FAA solution (formaldehyde: acetic acid: 70% ethanol, 1:1:20) for histomorphological inspection (samples are held at Ariel University). The exsiccata of all accessions are stored at Tel Aviv University (voucher specimen numbers from TELA4443 to TELA4450). Growth variation between male and female populations of wild grapevine accessions was examined through the assessment of variation in internode length and diameter using a digital caliper and a normal scale. This investigation encompassed three distinct male accessions and three distinct female accessions, measuring 3 branches for each plant, and ten internodes for each branch. The plants were collected from the Ariel University's Grapevine Germplasm collection, where plants are maintained at uniform growing conditions (common garden) to ensure uniform irrigation, soil, climate, and irrigation condition [25].

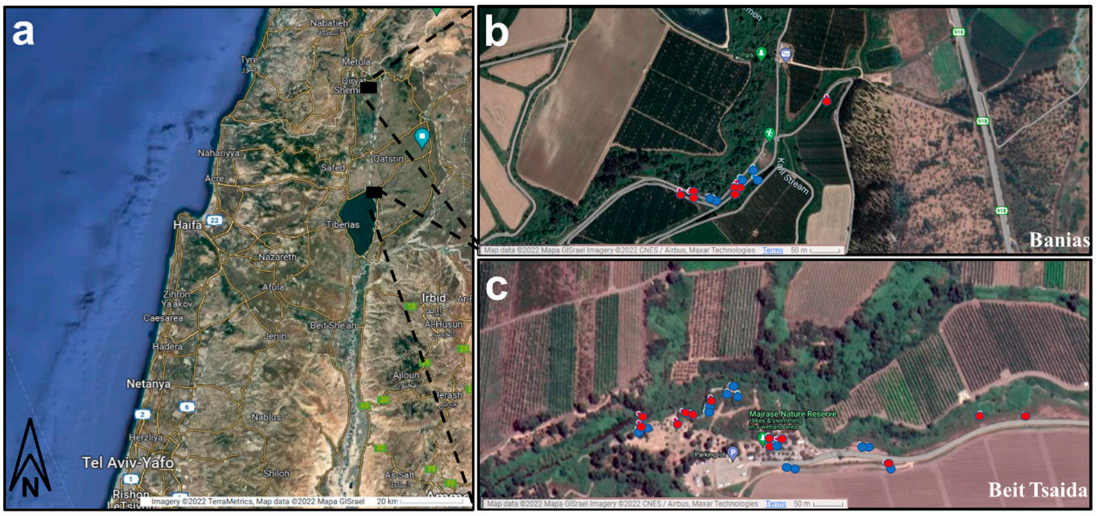

Figure 1. The study sampling sites (a) Satellite map of the northern part of Israel. The black squares indicate the location of two study areas on the map (b) An aerial photo of the Banias area (c) An aerial photo of the Beit Tsaida area. Blue and red dots represent male and female wild grapevine accessions, respectively. The map was created by Google Maps App. (Mapa GISrael).

Morphological characterization

Leaf: The leaf morphology was examined in dry herbarium specimens. We measured the length of the petiole, the length, and width of the leaves in their greatest extension, and calculated the ratio between length and width and the length ratios of the blade to the petiole. We noted the shade difference between the abaxial and adaxial sides of the leaf lamina, determining whether leaves are concolorous or discolorous. We described the leaf form according to the glossary in Kafkafi [26].

Seed and berry: To obtain healthy berries and seeds of optimal size for characterization, we studied plants that were cultivated under ideal conditions at the Ariel University's Grapevine Germplasm collection located at Ariel University. Approximately 60 seeds and 100 berries from each site were used for morphological characterization and statistics. The metric measurements (Berry diameter, seed length, and width) were taken using a Sparkfun electronics digital caliper (0-15 mm), and the data presented is the mean and standard deviation (Table 1). In addition, morphological descriptors (OIV)[27] were used to describe the morphological traits of seeds and berries.

Flower: Male and female flowers were placed on a microscope glass slide wrapped or unwrapped with black paper and illuminated with white LED light. Images were captured with a Nikon SMZ25 stereo-microscope (Nikon Ltd., Japan) equipped with a camera (Nikon DS-Ri2). Sixty digital photo-micrographs (resolution: 4908×3264 pixels), with each step about 50 μm, were taken at different focal planes and compiled to a single image using ND2-NIS elements software with an extended depth of focus (EDF) patch (Nikon Instruments, Japan). Images were then transformed into a single high-quality focused image using the dedicated software.

Pollen: Dried anthers with pollen grains were coated with gold using a sputter machine (Quorum Q-150T ES). The prepared samples were then imaged using FE-SEM (Tescan Ultra-High Resolution MAIA 3). Beam voltage 1.0 kV and SE detector were used for samples.

Tissue histology: Tissue samples of male and female flowers were fixed in FAA (formaldehyde: acetic acid: 70% ethanol, 1:1:20), embedded in paraplast, sectioned at 12-micron thickness with a rotary microtome (SLEE medical GmbH, Germany) and stained with safranin-alcian blue stain [28]. The slides were photographed under an Olympus SZX7 stereo-microscope equipped with a camera (Pixelink USB 3.0, Canada) and using PixeLINK Capture program.

Statistical analysis

Statistical analysis was carried out on each dataset to compute the mean, standard deviation, minimum, and maximum values. Subsequently, the normality of distribution was evaluated for every pair of parameters. In cases where a normal distribution was ascertained, a t-test was executed. Conversely, instances involving the identification of non-normal distribution were prompted by applying a Wilcoxon 2-sample test. A significant difference, defined by a p-value < 0.05, served as the basis for inferring equal variances. To explore the morphological attributes of leaf and petiole measurements, a Principal Component Analysis (PCA) was performed. All of the statistical analysis were performed using JMP® Pro 16.0.0 software [29].

3. Results and discussion

Sylvestris growth habit in wild habitats: In this study, we focused on the two sites where stable and large grapevine populations were previously reported [21]; the northern site at the Banias River, and the southern site at Beit Tsaida (Figure 1). Both sites are located on alluvial soils within the protected natural reserves (Banias and Majrase, respectively). Sampling was performed randomly along the water streams where grapevine grows. The sampling sites were chosen to be of equal size (circa 8000 m^2). In total, 46 accessions were collected, with 32 wild grapevine accessions originating from Beit Tsaida and 14 accessions from the Banias River. We scanned a similar area in both habitats, finding more individuals at the Banias than at Beit Tseida. The variation in population size of wild grapevine accessions between the Beit Tsaida and Banias River areas can likely be attributed to factors such as differing climatic conditions, nutrient availability, ecological characteristics, riverbank sizes, and varying levels of site disturbance by anthropogenic activities. Notably, the Banias River area has exhibited a reduction in species diversity due to pronounced anthropogenic activities.

Sampling was performed during the spring when plants were in full blossom which enables to identify developing pistils and stamens in dioecious (unisexual plants) or monoecious plants. All *Vitis* plants at both sampling sites were dioicous, supporting their identification as true Sylvestris. The ratio between male and female plants is close to 1, with a slightly higher number of male accessions (Table 1). This finding strengthens our assumption that the examined populations are wild, with no bias toward the fructiferous female form [30].

Growth habit varied between male and female grapevines at both locations. Male grapevines were taller and tended to climb to the tops of trees, while female grapevines were shorter and tended to prostrate in a tangle of low vegetation (Figure 2a, b). The male grapevines were characterized by multiple and densely packed flower clusters, usually located near the top of the vine (Figure 2b), and females produced fewer and smaller flower clusters (Figure 2c) which were located lower along the stem. These male climbing habit may be attributed to wind-pollination strategy [9] and the low stature of the female may be required to structurally support the heavy bunches of fruits. The sexual dimorphism observed in plants height and inflorescence position seem adaptive to the natural habitat.

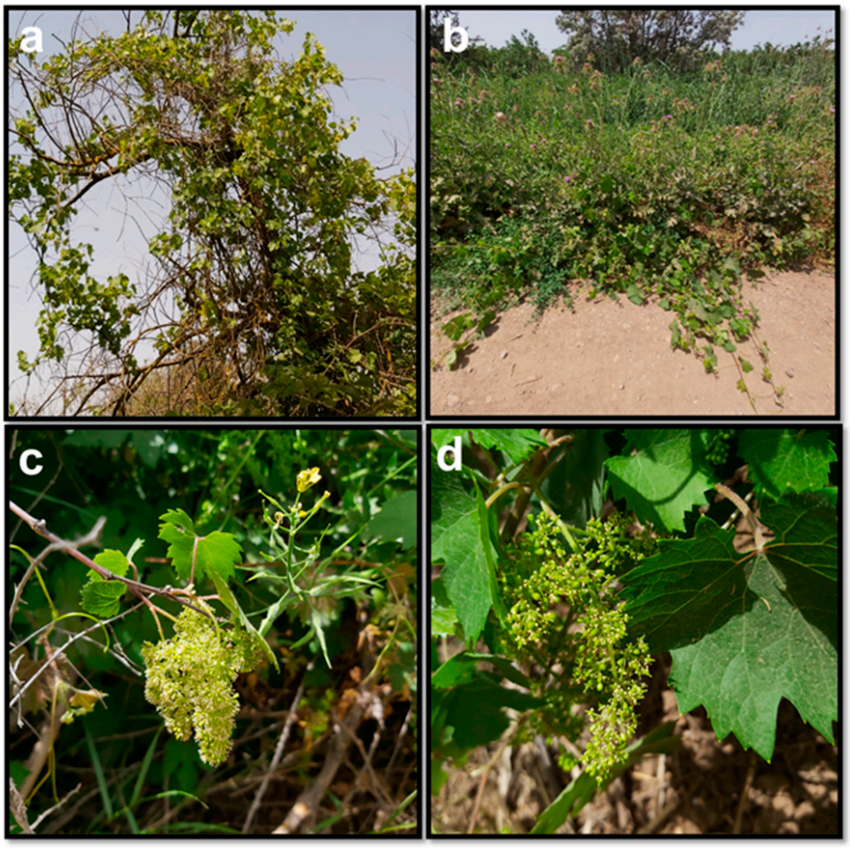

Figure 2. Growth habits of Sylvestris plants in Northern Israel. (a) Male grapevine climbs on the tree in Beit Tseida, (b) female grapevine by the side of the road in Banias (c) male inflorescence, d) female inflorescence.

In our current survey, grapevines were growing in deep uncultivated soils very close to flowing sweet water streams, as was previously reported [21]. Male and female plants were spread randomly. In both sites, the wild grapevines grow in proximity to fig trees (*Ficus carica*), plane trees (*Platanus orientalis*), and holy raspberries (*Rubus sanguineus*). This plant community is typical to water-rich habitats along the Mediterranean basin. Interestingly, the observed Sylvestris female plants tended to grow between the spiny holy raspberry plants which, apparently provide protection from herbivores that are abundant in these regions including the wild boar (*Sus scrofa*) [31]; mountain gazelle (*Gazella gazella*)[32] which commonly feed from grapevine shoots and leaves. Spiny vegetation, are supposed to have played an important role in the protection of wild vines from both wild and domestic animals [33,34]. On the other hand, male Sylvestris plants tend to climb on higher trees, plausibly, as means of support, avoidance of both herbivores, and competition for sunlight. Taller growth of male population has been supported by comparative observations of internode length and diameter of male and female accession growing in a common garden at our collection, where male plants have significantly longer internode length and wider stem diameter as compared to female accession (Figure 3).

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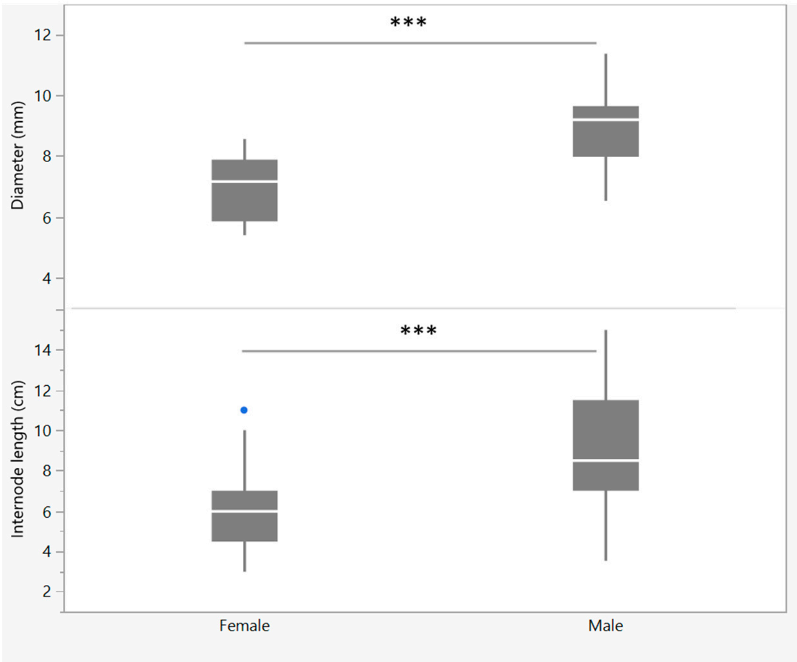

Figure 3. Comparison of the internode length (cm) and diameter (mm) of three male and female wild grapevine accessions maintained at Ariel University's Grapevine Germplasm collection. The horizontal white lines in the graph represent the median values. The boxes indicate the range between the 25th and 75th percentile of each group's distribution of values. Observations that fall outside this range are denoted by dots.

Overall, the observed populations at both sites have a distinctive appearance with a significant polymorphism in leaves, and clusters of small, greenish-yellow flowers developed into blackberries. Its growth habit and woody stem make it a hardy plant providing cover and habitat for various animals in its natural environment [35,36].

Morphology

Leaf morphology: Leaf shape and morphology show a high polymorphism, ranging from reniform with weak lobation to cordate with pronounced lobation. No significant correlation between leaf shape and sampling location was observed, and length-width blade ratios (p-value>0.05)) (Table 1). The blade-petiole length ratio was higher in Beit Tseida (p-value<0.001). The dorsal surface of the leaves from Banias was found to be hairy in contrast to leaves from Beit Tseida. This results in a shade contrast, causing the leaves in the Banias populations to be discolored, while the Beth Tseida populations have concolor leaves. Principal Component Analysis (PCA) conducted on the leaf data reveals a weak distinction among populations (Sup. Figure 1). These findings corroborate the outcomes derived from a greater number of OIV attributes in our prior study [21]. Based on the aforementioned genetic and morphological evidence, it is reasonable to conclude that the sampled populations do not represent discrete entities, but rather constitute a single population that has undergone adaptive changes in response to diverse environmental conditions across different geographic regions, thereby giving rise to specific morphological characteristics.

Table 1. Comparison of the main morphological features of the wild grapevines collected at both habitats. Terminology is according to [26], and to the OIV descriptors ([37]) for the berries and seeds.

	Beit Tseida	Banias
Abaxial/adaxial leaf color contrast	Concolor	Discolor
Blade/petiole length ratio	1.72 ± 0.92*	1.09 ± 0.3*
Blade shape	Not constant	Trilobate
Blade size ratio	0.8 ± 0.11^{ns}	0.76 ± 0.093^{ns}
Color of berry skin (OIV 225)	Blue black (6) #	Blue black (6) #
Berry shape (OIV 223)	Globose (2) #	Globose (2) #
Berry diameter [mm]	10.04 ± 0.90*	11.34 ± 0.97*
Seed shape	Ellipsoid	Ellipsoid
Seed length (OIV 242)	Very short (1) #	Very short (1) #
Seed length [mm]	5.38 ± 0.45*	5.10 ± 0.42*
Seed width [mm]	3.66 ± 0.29^{ns}	3.68 ± 0.23^{ns}
Observed ♀/♂ ratio	0.88	1

numbers indicate OIV description parameters [27]. * indicate significant differences between locations (p-value < 0.01); measured values are mean ± sd; ns is non- significant data.

Berry and seed morphology: Growing conditions have a dramatic effect on the number and size of grapes berries and seeds. To obtain healthy, optimal size berries and seeds for inspection and characterization cuttings were sampled from plants at both sites and were grown under optimal conditions at the Ariel University's Grapevine Germplasm collection at Ariel University [25]. Figure 4a shows well-grown and dense fruit bunches when grown under irrigation, while Figure 4b shows the sporadic bunches grown in the wild. A broad range of polymorphism in cluster shapes was observed among samples, yet most of them had sparse clusters of tiny, typically black berries, that usually contain 2-3 seeds (Figure 4c). The berries diameter was found to be significantly different (p-value < 0.001) between the populations when Banias Sylvestris berries being bigger than those of Beit Tseida (Table 1). In both populations, the berry's skin is thin, and the pulp is juicy and sweet with high acid levels, but lower than those found for European Sylvestris grapes [20]. The seeds length was differed significantly between sampling sites (t-test, p-value < 0.001), where the seeds from Beit Tsaida were larger (mean = 5.38, sd = 0.45)(Table 1). These values correspond to Sylvestris varieties as was previously recorded [38,39].

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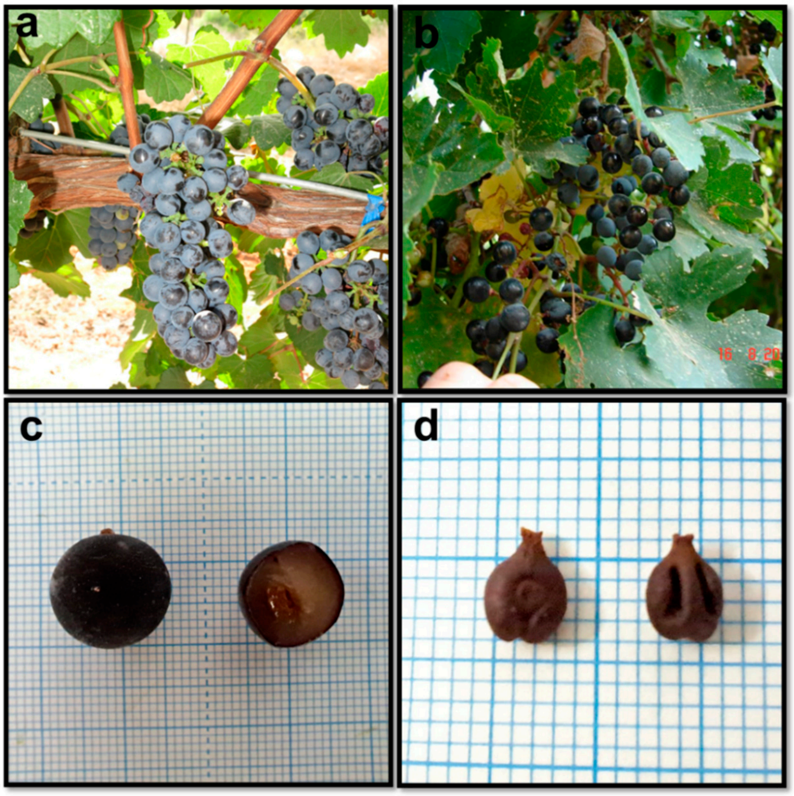

Figure 4. Berry and seed morphological traits of the wild grapevines collected from Northern Israel. Female grapevine bunches (a) from Ariel University's Grapevine Germplasm collection at Ariel University (b) from the wild (Beit Tsaida). (c) A whole grape berry next to a sectioned berry (Accession number 22, from Ariel University's Grapevine Germplasm collection). (d) Ventral (on the right) and dorsal (on the left) sides of seeds (Accession number 189, from Ariel University's Grapevine Germplasm collection) on graph paper of 1mm squares.

Flower morphology: It is well established that the main feature that distinguishes between wild and domesticated grapevine is the flower development and more specifically its reproductive organs [8]. The female flower includes an ovary and reflexed rudimentary/atrophied stamens that angle downwards, while the male flower displays upright stamens and a reduced pistil without style or stigma and an rudimentary/atrophied ovary at the base (Figure 5). These features are clearly distinctive to Sylvetris plants, while the Sativa forms, also found ocasionly growing feral, all have an hermaphrodite phenotype [20]. The flowering in the studied areas occurs in the month of May, giving rise to fruit on female individuals later in the summer (August). In our observations, the flowers of the wild grapevine are small and greenish-yellow and are arranged in panicles, the individual flowers have a diameter of around 5 mm and contain five petals fused at the base. Vitis vinifera sylvestris is the wild ancestor of the cultivated grapevine varieties Vitis vinifera [24]. In case of sylvestris the presence of both male and female plants in wild populations requires crossbreeding for the formation of fruit, while domesticated types are hermaphroditic and can self-pollinate [40]. The hermaphroditic nature of the Sativa types was an asencial part of the domestication proccess, leading to stable yields and full bunches due to better polination and the possibility of planting only reproductive plants without the need for male polinator plants [8].

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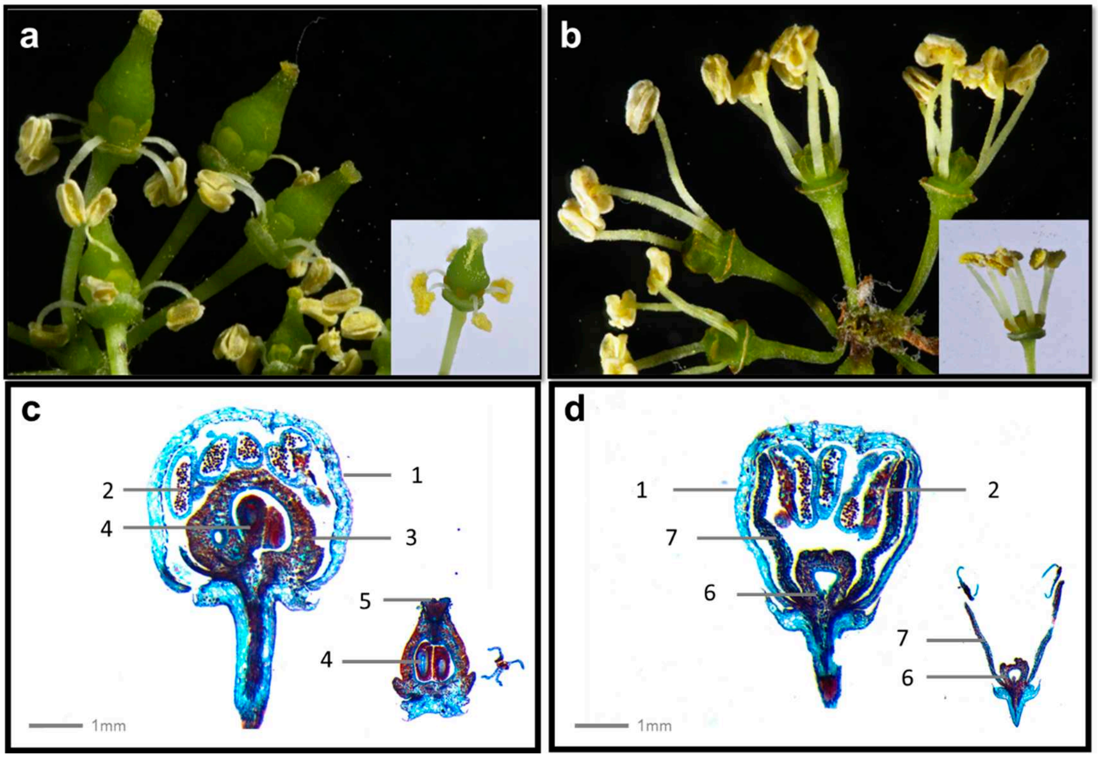

Figure 5. Female and male flowers of wild grapevines and their histological sections (a) Female and (b) male specimens at flowering. Histological sections of closed and open (on the low right side) (c) female and (d) male flowers. Parts of the flower: 1- Petal, 2-Anther, 3-Ovary, 4-Ovules, 5-Stigma, 6-Atrophied ovary remnant, 7-Anther filament.

The histological sections of the female grapevine flower show a single ovary (Figure 5c), a style, and a stigma, which comprise the pistil. The ovary is located at the base of the flower and contains ovules that will eventually develop into seeds if fertilized by pollen (Figure 5c). The structure of the male flower is distinctly different from that of the female flower (Figure 5). The male grapevine flower sampled at the same location had a different structure which consists of an atrophied ovary and stamens that are arranged in a tight cluster at the base of the flower. The stamens produce and release pollen grains from wind and/or insects to the female flowers, allowing for fertilization and fruit production [41].

Pollen grains morphology: A scanning electron microscope (SEM) was utilized to image the pollen of Sylvestris accessions sampled at experimental vineyard (Figure 6). In the male flowers, the pollen grains exhibited tricolpate morphology (with three furrows) and are ellipsoid in shape. In the female flowers, the pollen grains are inaperturate and spheroidal in shape (Figure 6). Additionally, pollen grains found on anthers in female flowers appeared collapsed or exhausted, and potent in male flowers further supporting dioecy. The morphological description of pollen grains in grapevines is consistent with previous findings on the differences between sterile and fertile pollen grains in Sylvestris [42,43].

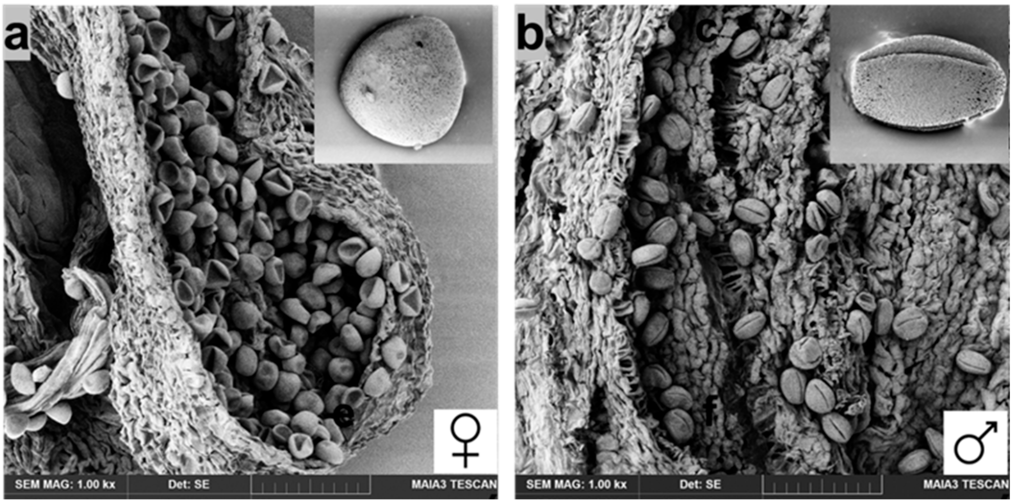

Figure 6. SEM images of pollen grains produced by (a) female and (b) male flowers of Wild grapevine (*Vitis vinifera* subsp. *sylvestris)* inside the anther. The scale bar is 50 μm enlarged single pollen grains are represented on the top-right of each image.

Natural seed germination: We conducted a thorough survey of the Sylvestris habitats in the wild in search for natural germination of seedlings. In the Banias area, we found five seedlings, all beneath female plants (Figure 7a). This germination habit was abundant indicating its success under the ecological conditions of this specific natural habitat. The young plantlets were carefully removed from the soil, including the remnants of their outer integuments (Figure 7c,d). The grapevine seeds were clearly identified despite being slightly damaged and soiled. To the best of our knowledge, this is the first time that natural germination of Sylvestris in its natural habitat has been recorded. These finds of natural germinations at a natural habitat provide strong evidence for the spontaneous nature of the population and its persistence strength as a stable population of Sylvestris – as an indigenous plant in Israel. Due to the growth habit of Sylvestris inside a dense bush of spiny raspberry plants we failed to identify young seedlings at the Beit Tseida area.

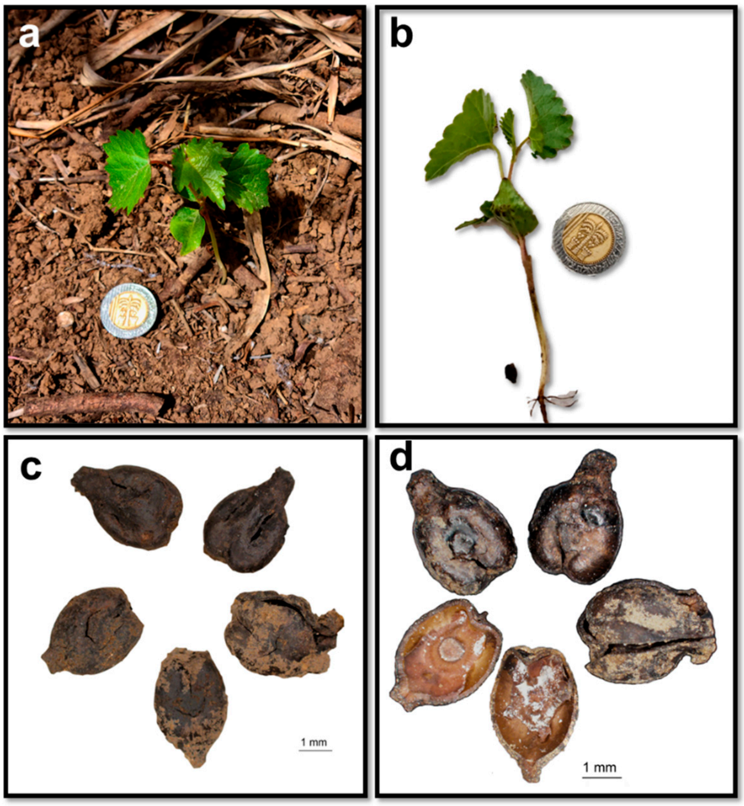

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Figure 7. *Vitis vinifera* subsp. *sylvestris'* germination in the field. (a) Naturally germinated grapevine seedling found in the Banias area of North Israel under female grapevines, and (b) the seedling following its extraction from the soil, including the remnant integument. (c,d) wild grape seeds (c-ventral side, d-dorsal side) which were carefully dug out and removed from the ground.

To summarize, previous records for grapevines growing in the wilds at the northern part of Israel, were considered to be feral Sativa plants, and there was no confidence as to the occurrence of *Vitis vinifera subsp. sylvestris* in Israel. The reasons were the lack of a comprehensive survey, and the minimal description of this population by the surveyors, giving only brief note on species presence [13,14]. Here, we systematically described the habitats, growth habits, morphology, and anatomy of widely spread wild grapevine populations growing in two distinct habitats. All of the above findings, including the dioecious nature of the wild grapevine plants, the sexual dimorphism between male and female plants, the characteristic traits of the flower development, pollen, berry size and morphology, seed structure, and the spontaneous regeneration of the population from seeds, together with our previous genomic findings showing clear separation of these populations from feral Sativa accessions [20,22], all lead us to the conclusion that natural wild grapevine populations grow in Israel. Our results indicate that wild grapevine occurs in natural habitats located within the region of its ancient area of appearance during the Pleistocene [24].

The high importance of this botanical description, clearing up any previous uncertainty as to the definition of these populations as Sylvestris, is due to the emerging importance of these wild populations as representatives of the core population from which the cultivated grapevine was first domesticated circa 11,000 years ago [24]. These facts also emphasize the significance of conservation of the environmental conditions and biodiversity of the Sea of Galilee and the forest habitats in the Upper Galilee, as the main habitats of this important populations.

Conclusions

The research presented here confidently supports the persistence of the wild grape species in the Israeli flora, extending the southern edge of its global distribution. As a result, Israel can be confidently added to the native distribution of *Vitis vinifera*, and this plant is included in the Flora Palaestina.

Author Contributions: "Conceptualization, OR; ED; SF; EW; methodology, OR; ED; IS; JZB; validation, OR; ED; IS; JZB; formal analysis, OR; JZB; investigation, OR; SF; ED; resources, ED; data curation, OR; JZB; writing—original draft preparation, OR; MMK; ED writing—review and editing, OR; JZB; IS; MMK; SF; SH; EW; ED; visualization, OR; MMK; ED; SF; supervision, ED; SH; project administration, ED; funding acquisition, ED All authors have read and agreed to the published version of the manuscript.".

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Conflicts of Interest: "The authors declare no conflict of interest."

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