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Sharing the Agrarian Knowledge with Archaeology: First Evidence of the Dimorphism of *Vitis* Pollen from the Middle Bronze Age of N Italy (Terramara Santa Rosa di Poviglio)

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Abstract: The recovery of inaperturate pollen from functionally female flowers in archaeological layers opens the question of a possible pollen-based discrimination between wild and domesticated *Vitis vinifera* in prehistoric times. Pollen analysis applied to archaeology has not routinely considered the existence of pollen dimorphism in *Vitis*, a well-known trait in the field of agrarian studies. Therefore, the inaperturate shape of grapevine pollen is ignored by studies on the archaeobotanical history of viticulture. In this paper we investigate pollen morphology of the domesticated and wild subspecies of *V. vinifera*, and report the first evidence of inaperturate *Vitis* pollen from an archaeological site. We studied exemplar cases of plants with hermaphroditic flowers, belonging to the subspecies *vinifera* with fully developed male and female organs, cases of dioecious plants with male or female flowers, belonging to the wild subspecies *sylvestris* and cases of *V. vinifera* subsp. *vinifera* with morphologically hermaphroditic but functionally female flowers. The pollen produced by hermaphroditic and male flowers is usually trizonocolporate; the pollen produced by female flowers is inaperturate. This paper reports on the inaperturate pollen of *Vitis* found in an archeological site of the Po Plain, Northern Italy. The site dated to the Bronze Age, which is known to have been a critical age for the use of this plant with a transition from wild to domesticated *Vitis* in central Mediterranean. Can the inaperturate *Vitis* pollen be a marker of wild *Vitis vinifera* in prehistoric times? Palynology suggests a possible new investigation strategy on the ancient history of the wild and cultivated grapevine. The pollen dimorphism also implies a different production and dispersal of pollen of the wild and the domesticated subspecies. Grapevine plants are palynologically different from the other Mediterranean "cultural trees". In fact, *Olea*, *Juglans* and *Castanea*, which are included in the OJC index, have the same pollen morphology and the same pollen dispersal, in wild and domesticated plants. In contrast, the signal of *Vitis* pollen in past records may be different depending on the hermaphroditic or dioecious subspecies.

Keywords: grapevine; pollen morphology; inaperturate pollen; cultivars; archaeological site; OJC; dioecy; domestication; reproductive biology; viticulture; Emilia Romagna

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1. Introduction

Vitis vinifera L. is a creeper species characterized by pollen dimorphism. This is well-known to scholars studying cultivars and vineyard productivity, but it is not generally known to palynologists working on the reconstruction of interlaced changes of environment and culture over millennia. This paper introduces the topic as a bridge between the agrarian expertise on modern crops and archaeobotany exploring proto-viticulture. The research points out that pollen dimorphism must be taken into consideration during palynological analyses and may be of help in discriminating between wild and domesticated plants, in the right contexts of the past.

1.1. Pollen Dimorphism

Pollen dimorphism, which means that two pollen morphotypes are present in one species, is a well-known feature of several Spermatophytes that may have a different origin. The causes of dimorphism can be found in an abnormal meiotic division of the pollen mother cell during the microsporogenesis, which results in pollen grains of different size and cytoplasmic content (in tobacco—*Nicotiana tabacum* L. [1]; in soybean cultivars—*Glycine max* (L.) Merril [2]). Pollen dimorphism may be associated to different stamen whorls of the flower (in crape myrtle—*Lagerstroemia* L. [3]), stigma dimorphism (Plumbaginaceae [4]) and heterostyly (Plumbaginaceae, *Turnera ulmifolia* L. [4,5]). Additionally, androdioecy and dioecy, or more exactly functional dioecy, can be responsible for pollen dimorphism (e.g., *Saurauia* Willd. [6–8]; *Thalictrum macrostylum* Small and A.Heller [9]). In functional dioecy, male plants produce normal pollen grains while functionally female flowers produce pollen grains which cannot fertilize, but generally act as an attractant for pollinators in nectarless flowers [6–9]. Complex causes are involved in the case of white campion—*Silene alba* (Miller) Krause [10].

Pollen dimorphism of *Vitis* is a case of functional dioecy and a known feature in several species (*V. riparia* Michx. [11]; *V. aestivalis* Michx. [12]; *V. coignetiae* Pulliat [13]) becoming an issue for the reproductive biology of the cultivated grapevine (*V. vinifera* L. subsp. *vinifera*).

1.2. The Botanical and Agrarian Knowledge

The dimorphism of grapevine pollen is connected to the morphology of flowers. Flower morphology of *V. vinifera* has been widely described and reviewed by Oberle [14], Pratt [15], Caporali et al. [16], Vasconcelos et al. [17], and Meneghetti et al. [18].

V. vinifera subsp. *sylvestris* (C.C.Gmel.) Hegi, wild grapevine, is dioecious. It derives from a hermaphroditic ancestor [19] and, interestingly, hermaphroditic flowers were observed together with male flowers from wild grapevines studied in Georgia (table 3 in De Lorenzis et al. [20]). Wild grapevine has hermaphroditic flowers that arrest one set of sexual organs very late during their development [21]. Fully developed male flowers producing trizonocolporate pollen show abortive pistils, whereas female flowers, albeit appear morphologically similar to the male ones, have a well-developed pistil but produce acolporate pollen in the stamens [16]. Inaperturate pollen is an important food source for pollinators often attracted by scent glands of flowers. The wild subspecies has an entomophilous and anemophilous pollination, and is the ancestor of the domesticated subspecies [20].

V. vinifera subsp. *vinifera*, the domesticated subspecies, has hermaphroditic flowers, which entailed changes in the reproductive process [22–24]. Actually, grapevine reverted to hermaphroditism during domestication [25,26], and is largely self-pollinating [20].

Indeed, some grapevine cultivars have morphologically hermaphroditic but functionally unisexual flowers. The consequence is that three types of flowers can be ascribed to the domesticated *V. vinifera* subsp. *vinifera*: one is the true hermaphroditic flower, with fully developed female and male organs producing trizonocolporate pollen, and two others are the morphologically hermaphroditic but functionally unisexual flowers, which are actually male flowers or female flowers [14,15,17,18]. Particularly, female flowers have a normally developed pistil surrounded by stamens, but filaments are more or less short, or curved and backwards reflexed [14,17,27]. This floral morphology is linked to the production of inaperturate pollen that is thought to be sterile [28,29]. Pollen sterility has been correlated to the lack of apertures (furrows and pores), which prevents pollen germination, and to post-meiotic disturbances [28,30] also determining poor viability of pollen [17].

Kevan et al. [11] suggest that pollen sterility of some cultivated varieties may be linked to the dioecy that characterized the wild progenitors of the domesticated subspecies. The inaperturate condition could be among the reasons why a dramatic drop of fruit yields can occur. Therefore, the consideration of this issue is of strategic importance in agrarian studies [31,32].

1.3. A Key Plant for Archaeology

Vitis vinifera subsp. *sylvestris*, the ancestor of the domesticated species [20,23], is a common creeper in riparian deciduous woodland and willow communities of Euro-Siberian and Mediterranean regions [33,34]. The domesticated *V. vinifera* subsp. *vinifera* has cultural implications and plays a substantial role in human economy being, in fact, among the most important crops and an emblem of Mediterranean agriculture, together with the olive tree.

As archaeologists give great attention to the history of grapevine and its role in past economy, impressive amounts of plant remains, including charcoals, fruits, and pips, are studied from archaeological layers [35–40]. Human selection for fruits caused changes in the morphology of seeds [41–44]. Therefore, the knowledge of the domestication history of grapevine largely relies on archaeological seeds, their discovery, morphology, biometry, chemistry, and molecular biology (mainly aDNA) [45–52]. Wild and domesticated traits of seeds are similar but different enough to allow discrimination of the subspecies with a certain degree of confidence [53,54].

In addition to these findings, also pollen is increasingly studied to follow the introduction and spread of the plant for cultivation, the uses to produce wine or vinegar, or for decoration in very different contexts (see, for example, the pergola discovered in a Roman villa in Sicily [55]). Pollen analysis has demonstrated that the trizonocolporate *Vitis* pollen is underrepresented or rare in modern contexts [56], as well as in many archaeological samples. Actually, it may be an indicator of the local presence of viticulture [57].

Nevertheless, pollen analysis applied to archaeology did not yet consider the dimorphism of the grapevine pollen and has ignored its inaperturate shape. This trait, instead, is well known in viticulture, because of implications on vineyard yield.

In this paper we introduce the first records of inaperturate *Vitis* pollen observed in samples taken from archaeological layers. The case study is a Middle and Late Bronze Age site—the Terramara Santa Rosa di Poviglio, which has been studied from a geoarchaeological and palaeoenvironmental point of view to reconstruct the sylvo–agro–pastoral system of this culture. There is no local evidence of grapevine records as plant macroremains while trizonocolporate *Vitis* pollen was observed in many samples [58–61]. The hypothesis is that the inaperturate *Vitis* pollen, though rare, can be a supportive evidence of the presence of wild female grapevine plants blooming close to the study area (see below, Section 4.3).

2. Materials and Methods

2.1. Reference Pollen Samples

Reference pollen samples were prepared for morphological observation collecting flowers from four cultivars (=cv.) of *Vitis vinifera* subsp. *vinifera* and from accessions of *V. vinifera* subsp. *sylvestris*, three grown in a germplasm collection, and male and female plants observed close in the wild (Table 1).

2.1.1. *V. vinifera* Subsp. *vinifera*

The inflorescences were taken from vineyards cultivated in the Emilia Romagna region (Northern Italy). Lambrusco Grasparossa and Malbo Gentile are regional red grape varieties mainly cultivated in the provinces of Modena and Reggio Emilia. Covra is a very rare red grape variety probably native of the same area. Bianca di Poviglio, from Poviglio (Reggio Emilia), is a local synonym of Trebbiano Toscano, well known white grape cultivar also widely cultivated in France, with the name of Ugni Blanc. All the sampled cultivars have an ancient origin and are described in the Italian Vitis Database ([62], and see below).

Table 1. List of reference pollen samples, location and date of sampling.

Sample	Species	Subsp.	Cultivar/ Label	Flower Morphology	Flower Functionality	Location	Date
1	*Vitis vinifera*	*vinifera*	Covra	Hermaphroditic	Male/female	Corlo di Formigine (MO)	May 2014
2	*Vitis vinifera*	*vinifera*	Lambrusco Grasparossa	Hermaphroditic	Male/female	Corlo di Formigine (MO)	May 2014
3	*Vitis vinifera*	*vinifera*	Bianca di Poviglio	Hermaphroditic	Male/female	Reggio Emilia (RE)	May 2016
4	*Vitis vinifera*	*vinifera*	Malbo Gentile	Hermaphroditic	Female	Albinea (RE)	May/ June 2009
5	*Vitis vinifera*	*sylvestris*	Accessione Canalone Sotto	Abortive pistil—fully developed stamens	Male	Torrazza Coste (PV)	May 2016
6	*Vitis vinifera*	*sylvestris*	Accessione Ponte 5	Abortive pistil—fully developed stamens	Male	Torrazza Coste (PV)	May 2016
7	*Vitis vinifera*	*sylvestris*	Accessione Pioppetto 3	Hermaphroditic	Female	Torrazza Coste (PV)	May 2016
8	*Vitis vinifera*	*sylvestris*	Wild dioecious plants	Abortive pistil—fully developed stamens	Male	Sammontana (FI)	June 2019, 2020
9	*Vitis vinifera*	*sylvestris*	Wild dioecious plants	Hermaphroditic	Female	Sammontana (FI)	June 2019

2.1.2. *V. vinifera* Subsp. *sylvestris*

The flowers of three accessions (labelled "Accessione" in Table 1) were taken from the germplasm collection vineyard established in 2006 at the Regional Research Station of Riccagioia in Lombardy (Northern Italy). These accessions had been identified and collected in Tuscany and are listed in the European Vitis Database [63]. Their flowers were drawn, and photos were taken with Leica MC120 HD under a stereomicroscope Leica Wild M10 (Figure 1). The other specimens come from "wild dioecious plants" growing at Sammontana, Florence (43°43′27.87″ N; 11°00′39.70″ E; 42 m a.s.l.), a registered station of Wild Grapevine of the Phytogeography Laboratory of the University of Florence, in Tuscany.

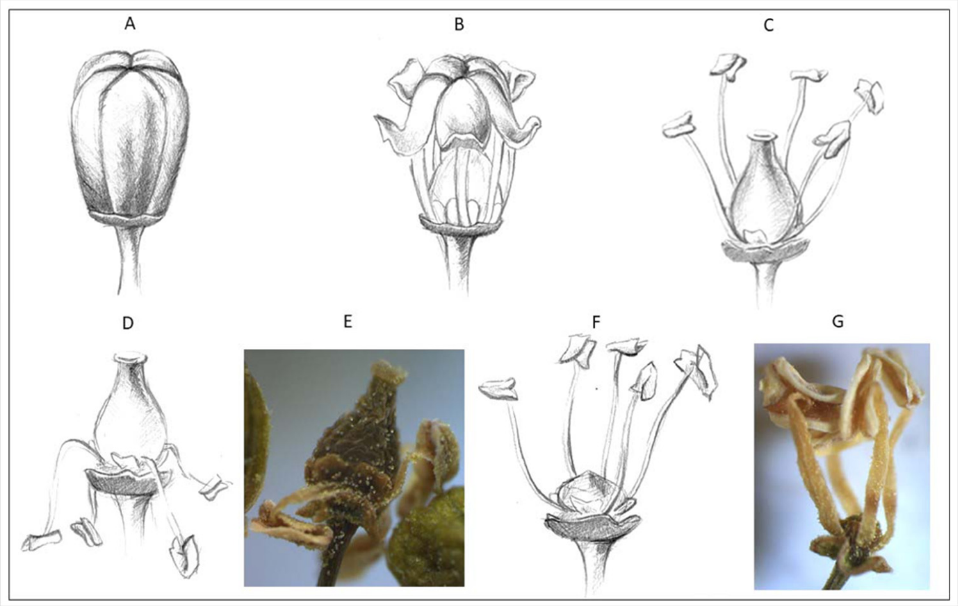

Figure 1. Hermaphroditic flower of *Vitis vinifera* subsp. *sylvestris*. (**A**) The petals are sticking together as a cap at the tip (calyptra); (**B**) the calyptra is shed explosively; (**C**) flower with the pistil and five stamens; (**D**) functionally female flower; (**E**) Pioppetto 3 accession (functionally female); (**F**) functionally male flower; (**G**) Canalone Sotto accession (functionally male) ((**A**–**D**,**F**): drawings by Alice Montanari; (**E**,**G**): photos made under stereomicroscope).

2.1.3. Acetolysis and Morphometry

The anthers of all samples were subjected to dehydration followed by acetolysis [64], and pollen slides were mounted in glycerol/water 50/50 *v*/*v*. The morphological parameters of trizonocolporate pollen were (a) equatorial view: polar axis (P), equatorial diameter (E), P/E ratio, maximum distance between colpi in mesocolpium (MES), polar and equatorial axes of porus (P-porus, E-porus); and (b) polar view: equatorial diameters (E1 and E2) and exine thickness (Ex). The inaperturate pollen has not a clear polarity, as any aperture location could be related directly to pollen polarity. In this case, the considered morphological parameters were the largest diameter (DM), smallest diameter (dm), DM/dm ratio, and exine thickness (Ex).

Pollen grains were measured according to Wrońska-Pilarek et al. [65]. The principal component analysis (PCA; XLStat 2014) was performed after data standardization by using the average values of the pollen grain size parameters as variables [66]: one PCA was performed with all the samples using P, E, and P/E ratio of the trizonocolporate pollen as variables; another analysis was performed with the variables obtained from inaperturate pollen (DM, dm, and DM/dm ratio), including the archaeological sample.

2.2. *Pollen Viability and Germination*

Considering the role of morphology in the viability of pollen, tests on viability and germination were performed on pollen of *Vitis vinifera* cv. Malbo Gentile almost totally inaperturate. Pollen grains were collected from capped flowers (from which calyptra was removed by fine forceps) and flowers at capfall. Viability was verified applying the Fluorochromatic Reaction Test, according to Heslop-Herrison & Heslop-Herrison [67] and Shivanna and Rangaswamy [68], using fluorescein diacetate (FDA) solution. Pollen was observed under a light microscope (LM) (Orthomat, Leitz) equipped with a Leica fluorescence illuminator (filters: BP 420-490, RKP 510, LP 515). The test was carried out observing three microscope slides, each with at least 1000 pollen grains.

To check in vitro germination, 1 mg pollen grains was incubated at 30 °C in the dark for 30, 60, and 90 min, in 1 mL of a liquid culture medium containing 10% sucrose, 0.02% $MgSO_4$, and $CaCl_2$, 0.01% KNO_3 and H_2BO_3. The samples were prepared on microscopic slides at the end of each incubation time and observed under LM in order to record germ tubes emission.

2.3. Scanning Electron Microscopy

SEM observations of inaperturate pollen were independently made on fresh and acetolyzed pollen grains. The acetolyzed pollen of the Wild dioecious plant (sample no. 8) was washed in acetic acid and ascendant series of acetic acid and ethanol, dried at Critical Point Dryer using CO_2 as the transition liquid. The samples were mounted on aluminium stubs, gold-coated (10 nm thick), and observed with ZEISS EVO MA15 (MEMA—University of Florence).

Flowers of *Vitis vinifera* cv. Malbo Gentile (sample no. 4), were conventionally fixed in glutaraldehyde (3% in 0.1 M phosphate buffer pH 7.0 at 4 °C), rinsed in the same buffer, dehydrated in ascendant acetone series, and dried at Critical Point Dryer using CO_2 as the transition liquid. Samples were mounted on aluminium stubs, gold-coated (10 nm thick), and observed with a Philips XL 30 Scanning Electron Microscope at CIGS (Interdepartmental Large Instruments Center—University of Modena and Reggio Emilia).

2.4. The Archaeological Record for Pollen Analyses

Cremaschi et al. [60] report the pollen extraction from sediments, and the counts and results on paleo-environmental reconstruction and land-use. A total of 31 archaeological samples were treated for pollen extraction. They were labelled Poviglio P31 to P1, from the oldest to the most recent. Laboratory treatments included sieving with a nylon 7 µm mesh and heavy liquid separation [69]. Pollen identification was made at 400× and 1000× magnification on permanent slides. For this study, the same permanent slides where trizonocolporate pollen was observed, were read again to search for inaperturate pollen grains. The sample Poviglio P19 contained inaperturate pollen that were subjected to morphometrical observations. Morphometric parameters of the *Vitis* pollen were measured: in the trizonocolporate pollen, polar axis (P), equatorial diameter (E), P/E ratio and exine thickness (Ex); in the inaperturate pollen, larger diameter (DM), smaller diameter (dm), and exine thickness (Ex).

3. Results

3.1. Pollen Morphology of Reference Pollen Samples

A total of 262 trizonocolporate and 120 inaperturate pollen grains were measured. The observed ranges are summarized and reported as mean value of each parameter measured over a minimum of 30 pollen grains per sample (Tables 2 and 3).

Trizonocolporate pollen was observed in all the samples belonging to hermaphroditic or functionally male flowers. The maximum values were measured in the *Vitis vinifera* subsp. *sylvestris* accessions.

Table 2. Morphometric data of the inaperturate pollen grains taken from functionally female flowers and from the archaeological sample (measurements under light microscope). Note: Each value is the average of the measurements ± standard deviation. Ex, exine thickness; DM, largest diameter; dm, smallest diameter; Pollen shape: 0, suboblate; 1, oblate spheroidal; 2, spheroidal; 3, prolate spheroidal; 4, subprolate; 5, prolate; Exine, ornamentation: 0, microreticulate; 1, scabrate-verrucate; 2, fossulate.

Sample	Cultivar/ Label	No of Pollen Grains	Ex (μm)	DM (μm)	dm (μm)	DM/ dm	Pollen Shape (%)						Exine
							0	1	2	3	4	5	
4	Malbo Gentile	30	1.2 ± 0.10	27.1 ± 1.70	25.1 ± 2.20	1.08	-	10	17	47	23	3	1
7	Accessione Pioppetto 3	30	1.3 ± 0.13	25.4 ± 1.35	25.3 ± 1.12	1.00	-	37	10	-	53	-	0
9	Wild dioecious plants	45	1.2 ± 0.35	21.9 ± 0.9	20.3 ± 1.5	1.08	-	-	13	67	20	-	2
Poviglio P19	-	15	1.1 ± 0.15	25.7 ± 4.6	22.5 ± 3.5	1.14	-	-	4	38	42	16	0

Table 3. Morphometric data of the trizonocolporate pollen grains taken from cultivated grapevine, male wild vines and the archaeological sample P19 (measurements under light microscope). Note: Each value is the average of the measurements ± standard deviation. P, polar axis; E, equatorial diameter; MES, maximum distance between colpi in mesocolpium; P-porus, polar axes of porus; E-porus, equatorial axes of porus; Ex, exine thickness; E1–E2, equatorial diameters; Pollen shape: 0, suboblate; 1, oblate spheroidal; 2, spheroidal; 3, prolate spheroidal; 4, subprolate; 5, prolate; Exine, ornamentation: 0, microreticulate; 1, perforate.

Sample	Cultivar/ Label	No of Pollen Grains	Equatorial View							Polar View		Pollen Shape (%)						Exine
			P (μm)	E (μm)	P/E	MES (μm)	P-Porus (μm)	E-Porus (μm)	Ex (μm)	E1 (μm)	E2 (μm)	0	1	2	3	4	5	
1	Covra	30	22.5 ± 1.34	20.7 ± 1.38	1.09	9.8 ± 1.06	1.7 ± 0.26	1.5 ± 0.19	0.9 ± 0.10	17.8 ± 1.14	20.4 ± 0.70	-	7	3	70	20	-	0
2	Lambrusco Grasparossa	30	20.7 ± 1.65	20.5 ± 1.62	1.01	10.3 ± 1.33	2.1 ± 0.33	1.7 ± 0.33	0.8 ± 0.10	20.9 ± 1.03	22.7 ± 0.93	3	47	6.5	37	6.5	-	0
3	Bianca di Poviglio	30	23.6 ± 2.10	23.6 ± 2.19	1.00	9.8 ± 1.80	1.8 ± 0.31	1.6 ± 0.25	0.9 ± 0.16	21.4 ± 2.06	24.7 ± 2.09	6	53	-	37	4	-	0
5	Accessione Canalone Sotto	30	24.3 ± 1.43	22.9 ± 1.10	1.06	12.5 ± 0.89	1.7 ± 0.29	1.5 ± 0.28	0.9 ± 0.10	18.3 ± 1.02	21.1 ± 1.05	6	14	-	74	6	-	0
6	Accessione Ponte 5	30	24.9 ± 1.90	22.8 ± 1.22	1.09	10.4 ± 1.20	1.5 ± 0.23	1.4 ± 0.20	0.8 ± 0.08	20.4 ± 1.28	22.8 ± 1.31	-	3	-	83	14	-	0
8	Wild dioecious plants	82	21.2 ± 3.66	20.5 ± 4.20	1.03	13.2 ± 3.80	2.6 ± 1.60	2.1 ± 1.30	1.6 ± 0.70	18.8 ± 2.70	20.5 ± 3.40	1	11	4	62	21	1	0/1
Poviglio P19	-	30	23.6 ± 3.90	15.8 ± 2.50	1.49	-	-	-	0.9 ± 0.12	-	-	-	-	-	-	6	94	0

Polar axis (P) ranges from ~20.7 µm (cv. Lambrusco Grasparossa) to ~24.9 µm (Ponte 5 accession); Equatorial axis (E) ranges from ~20.5 µm (cv. Lambrusco Grasparossa, cv. Covra, Wild dioecious) to ~23.6 µm (cv. Bianca di Poviglio). The mean values of the P/E ratio are mostly ~1/1.1, and the pollen grains are suboblate to prolate in shape (terminology according to Erdtman [70]; Figure 2). The maximum distance between colpi in mesocolpium (MES) ranges from ~9.8 µm (cv. Covra, cv. Bianca di Poviglio) to ~12.5 µm (Canalone Sotto accession) and ~13.2 (Wild dioecious); the porus diameter ranges from ~1.4 µm (Ponte 5 accession) to ~2.6 µm (Wild dioecious). Exine thickness (Ex) is thin in the present and in the archaeological specimens (0.8–0.9 µm) with the only exception of the Wild dioecious that is ~1.6 µm thick. Exine sculpturing looks slightly micro-reticulate under light microscopy and micro-rugulate at SEM (Figure 3).

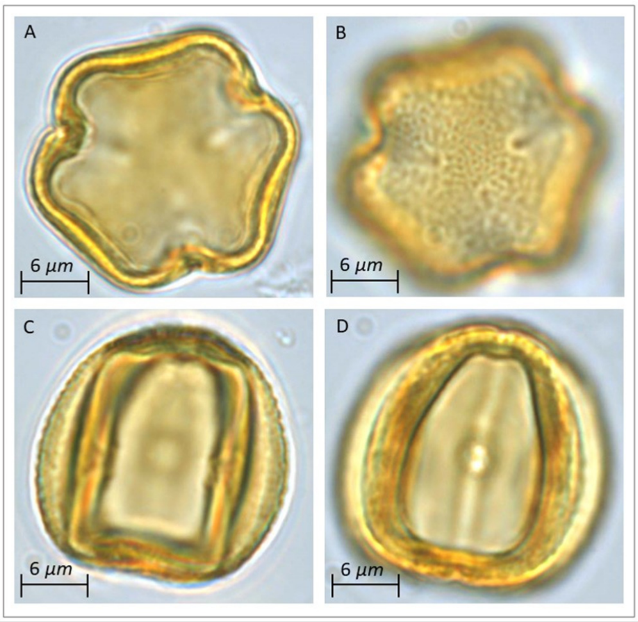

Figure 2. Light microphotographs of trizonocolporate pollen of *Vitis vinifera* cv. Bianca di Poviglio. (**A**) polar view, with the exine thickness in focus; (**B**) polar view, with the upper surface in focus showing the exine reticule; (**C**) equatorial view with two colpora in focus; (**D**) equatorial view with the lower surface in focus showing the third colporus.

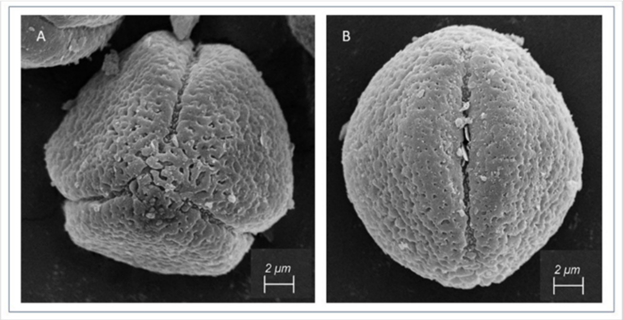

Figure 3. Scanning electron microphotographs of trizonocolporate pollen of Wild dioecious functionally male flower (**A**) in polar view; (**B**) in equatorial view.

Inaperturate pollen was observed in the samples belonging to functionally female flowers of the two specimens of the female accessions of *V. vinifera* subsp. *sylvestris* and in the cv. Malbo Gentile. The maximum values were measured in the cv. Malbo Gentile (Table 2; Figure 4A,B). DM ranges from ~21.9 µm (Wild dioecious) to ~27.1 µm (cv. Malbo Gentile), and dm ranges from ~20.3 µm (Wild dioecious) to ~25.0 µm (cv. Malbo Gentile; Pioppetto 3 accession). The mean values of the DM/dm ratio are mostly ~1/1.2, and the pollen grains are from oblate-spheroidal to prolate in shape. Exine thickness (Ex) is thin (1.1–1.3 µm) but slightly thicker than in the trizonocolporate pollen.

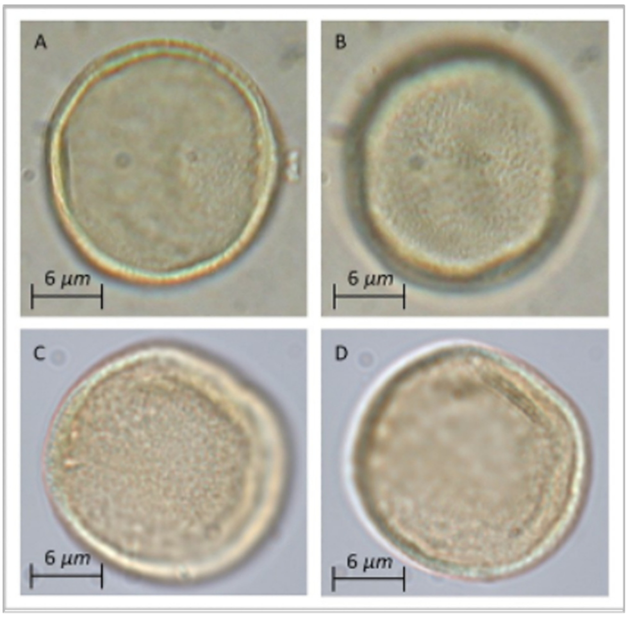

Figure 4. Light microphotographs of inaperturate pollen grains. (**A**) *Vitis vinifera* cv. Malbo Gentile; (**B**) image of the same pollen grain taken at different focus distance; (**C**) *Vitis* in the archaeological sample Poviglio P19; (**D**) image of the same 'archaeological' pollen taken at different focus distance.

Under light microscopy exine sculpturing looks slightly micro-reticulate in Pioppetto 3 accession, micro-rugulate in cv. Malbo Gentile and in Wild dioecious. Under SEM exine sculpturing looks micro-rugulate (Figure 5).

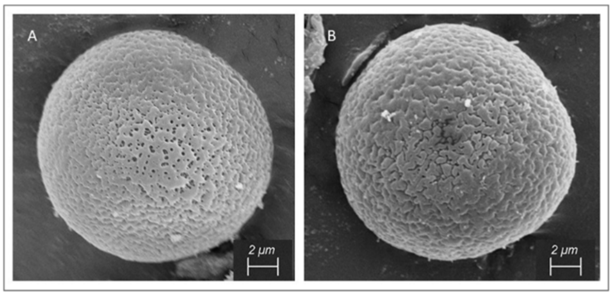

Figure 5. Scanning electron micrographs of Wild dioecious plant. (**A**) Inaperturate pollen grain of functionally female flower; (**B**) image of the same pollen grain taken at different focus distance.

3.2. *Pollen Viability*

Only 4.5% of pollen of cv. Malbo Gentile was observed with apertures. The result of pollen viability test on inaperturate pollen was positive, with 92.4% (±6.1) of pollen tube grains viable (Figure 6). In vitro germination and pollen growth were not observed, even after 90 min of incubation. This result of the in vitro germination test, associated with the high percentage of pollen resulting positive under the Fluorochromatic Reaction Test, allows excluding the possibility that cv. Malbo Gentile produces some cryptoaperturate pollen (sensu Punt et al. [71]). Indeed, this suggests that pollen sterility is due to the inability to let out pollen tube [29], in agreement with scientific literature [32,72]. The same tests are currently applied to trizonocolporate pollen, i.e., in *Vitis vinifera* cv. Lambrusco Salamino, obtaining positive results also in pollen germination [73].

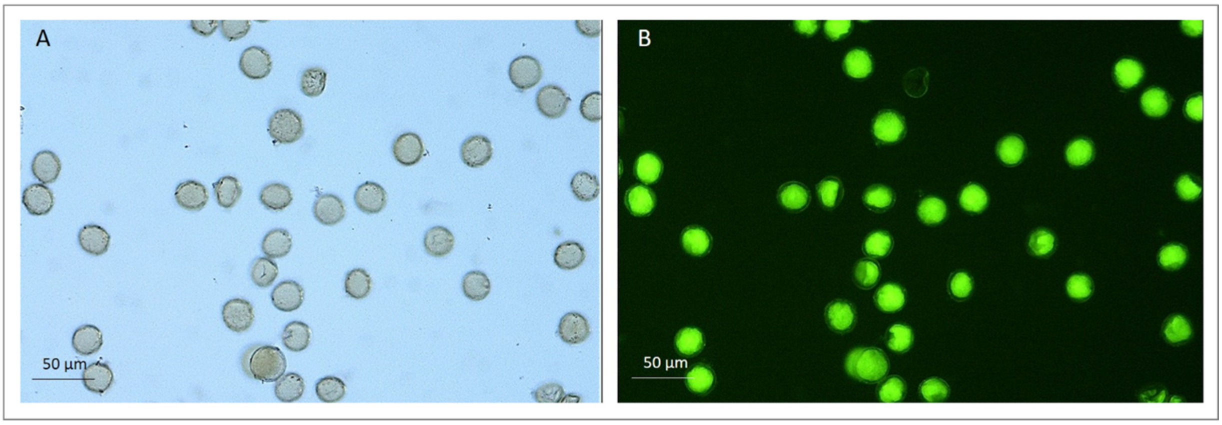

Figure 6. Results of viability test on pollen of *Vitis vinifera* cv. Malbo Gentile. (**A**) light microphotograph showing inaperturate pollen grains; (**B**) the same sample observed under UV light. All viable pollen grains show intense bright green fluorescence.

3.3. Pollen from Archaeological Samples

Inaperturate pollen was observed also in the samples taken from the archaeological site. Previous results from pollen analyses have shown that *Vitis* pollen, detected in 87% of the samples, reached 3% on average [60]. The maximum value of 18%, consisting of trizonocolporate pollen, was counted in sample P19, dated to the Middle Bronze Age. Interestingly, the re-examination of the slides allowed counting also 15 inaperturate pollen grains in the same sample.

Regarding morphology, the trizonocolporate pollen looked more prolate than the reference records (Table 3; Figure 7), showing a large P = ~23.6 µm but a decidedly low E = ~15.8 µm. This results in a relatively high P/E = 1.49, i.e., prolate shape (Tables 2 and 3). The inaperturate pollen has diameters of ~25.7 × 22.5 µm, quite similar to the largest measurements observed in the reference pollen of cultivated plants (Figure 4C,D). Under light microscopy exine sculpturing looks slightly micro-reticulate like in Pioppetto 3 accession.

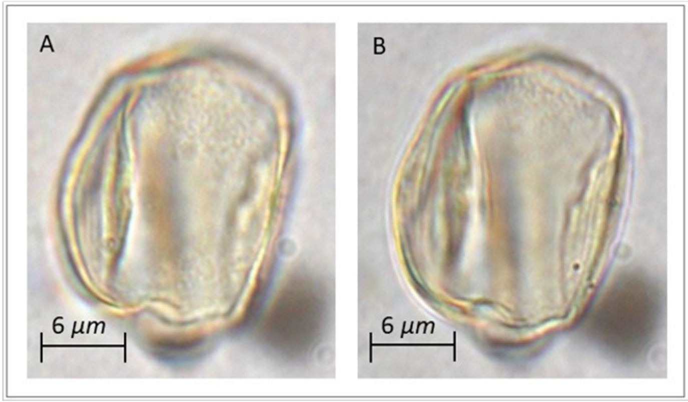

Figure 7. Light microphotographs of *Vitis* trizonocolporate pollen grain in equatorial view in archaeological sample Poviglio P19. (**A**) *Vitis* pollen grain with the upper surface in focus; (**B**) the same pollen grain at a different focus, showing the colpora features.

4. Discussion

The record from the Terramara Santa Rosa di Poviglio represents the first finding of inaperturate pollen of wild grapevine female plants observed in an archaeological site. Pollen data suggest the compresence of male or hermaphroditic *Vitis* plants (producing trizonocolporate pollen) and female plants (producing inaperturate pollen) growing in the site.

The evidence opens the question about a possible pollen-based discrimination between wild and domesticated *Vitis vinifera* in prehistoric times. Can the inaperturate pollen be associated with the growth of wild species in a study area? Can this record be considered an interesting marker of wild *Vitis* in archaeobotanical analyses? Based on the pollen morphology of modern specimens of *Vitis vinifera*, the archaeological evidence may be compatible with the presence of both wild dioecious vines or some domesticated grapevines living close to the deposit studied.

Therefore, the positive answers to the above-mentioned questions cannot be based only on the pollen evidence. It depends also on the availability of other archaeobotanical data (fruits and pips remnants, morphology of the seeds), which are often available from archaeological sites and can integrate this information. However, as we know, macroremains of *Vitis* have not been found so far while pollen is quite common in the Bronze Age samples from the Terramara Santa Rosa di Poviglio [60]. The pollen evidence can be of particular importance in case of lack of macroremains. Furthermore, the detection of an increase of inaperturate pollen grains overtime (centuries and millennia) could be a sign of a trend towards a conscious use of female wild vines near the settlements [74], which could be regarded as a typical step of proto-viticulture.

4.1. Pollen Dimorphism of Current Vitis vinifera

Observation of pollen morphology confirms that trizonocolporate and inaperturate pollen grains can occur in both the *Vitis* subspecies in this study.

In the PCA of trizonocolporate pollen parameters (P, E, P/E; Figure 8A) the two principal components are responsible for 61.74% and 38.18% of the total variation. The distribution shows that samples with the highest values of P and E are grouped in the second sector of the PCA. Pollen samples are distributed along the second principal component reflecting the size of polar axis (P) and showing a clear clustering of both the *V. vinifera* subsp. *sylvestris* accessions (Canalone Sotto and Ponte 5) and the pollen from ancient varieties of *V. vinifera* subsp. *vinifera* (cv. Bianca di Poviglio). Only Wild dioecious is quite distinct from other samples of the same subsp. *sylvestris*. The archaeological sample (P19) is clearly distinguished from the reference pollen samples through a grain size-based gradient.

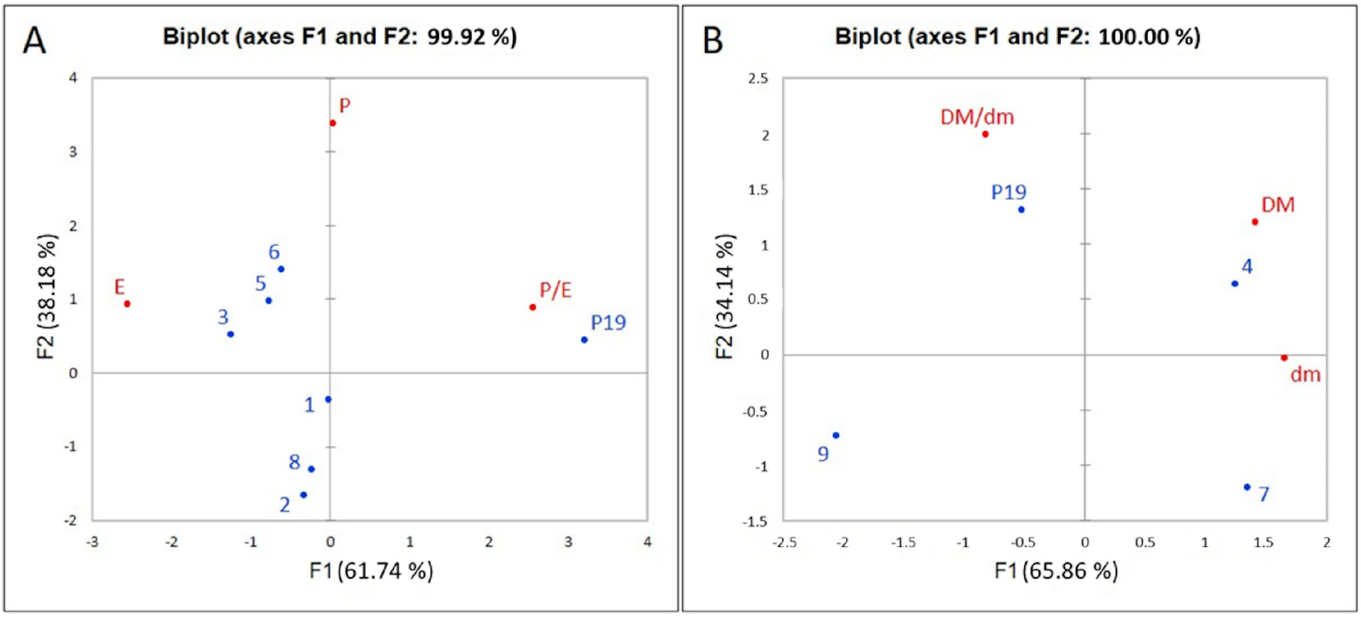

Figure 8. Principal component analysis. (**A**) Trizonocolporate pollen grain size parameters (P, polar axis; E, equatorial diameter; P/E ratio); (**B**) Inaperturate pollen grain size parameters (DM, largest diameter; dm, smallest diameter; DM/dm ratio). Names of samples are labelled following codes in Table 1.

The two main axes of the PCA processing inaperturate pollen grain size parameters (DM, dm and DM/dm; Figure 8B) explain 65.86% and 34.14% of the total variation, respectively. The distribution of the samples is independent and does not show clustering based on the variables considered.

4.1.1. Trizonocolporate Pollen

Usually, hermaphroditic and male flowers produce pollen grains that are morphologically similar in the two subspecies since they are all trizonocolporate and characterized by micro-reticulate structure of the exine. This morphology is in accordance with Punt et al. [75] and Gallardo et al. [76]. Pollen has a quite rounded shape (P/E = ~1). Particularly, the pollen from ancient varieties of *V. vinifera* subsp. *vinifera* (cv. Bianca di Poviglio) looks quite similar in size/shape to the pollen of the subspecies *sylvestris*. The significant similarity could be related to the domestication process, and the transition from unisexual to hermaphroditic flowers.

In the archaeological record the shape (P/E = 1.49) is decidedly more prolate than in modern pollen grains. This difference is most probably due to post-depositional processes and related to the fact that once incorporated in sediments pollen undergoes different degrees of compaction effects. The sub-fossil, archaeological, pollen has a distorted, folded or partially flattened, shape.

4.1.2. Inaperturate Pollen

During pollen analyses, this morphology can be associated with that of similar pollen or palynomorphs from which, however, diagnostic discrimination is possible. Inaperturate pollen is common in Gymnosperms and characterizes many Angiosperm groups. Presence of orbiculi (e.g., *Cupressus*) and exine thickness are important traits for identification. Size overlapping may be observed with the spores of Bryophyta, which range from 7 to 8 µm up to 40 to 50 µm, but the exine thickness with more or less irregular processes—determining a more diaphanous/yellow colour of the spore—is quite different from that of pollen in the same samples. Further, the pollen of *Populus* has a pretty similar morphology. In the different species, this is inaperturate and ranges from 16 to 37 µm in diameter, whose size partly overlaps with that of the inaperturate pollen of *Vitis* (in the range of 22–27 µm). However, *Populus* pollen has extremely thin exine (0.7–1.0 µm [77]), with granules or microgemmae irregularly sparse, while the *Vitis* inaperturate pollen has slightly thicker exine (1.0–1.3 µm) with more regular pattern of the surface wall. In *Populus*, the reduction of the exine thickness through which the pollen tube can easily exit may be related to its wind-pollination. This pollination strategy does not require the presence of large cavities in the wall for the storage of the pollenkitt and it is favored by a reduction of the exine amount, which makes pollen more aerodynamic [8].

In our study, direct pollen examination confirms that the female flowers of subspecies *sylvestris* Pioppetto 3 and the Wild plants, as a result of the dioecious status, produce inaperturate pollen. However, this special character is observed also in cv. Malbo Gentile of the subspecies *vinifera*, where viability and germination tests support the hypothesis that the absence of colpora may be responsible of sterility in this cultivar. A gene, named INAPERTURATE POLLEN1 (INP1), specifically involved in the formation of pollen apertures, has been recently isolated and characterized in *Arabidopsis thaliana*; loss of INP1 entails the lack of furrows and INP1 protein shows an exact localization in developing pollen [78]. Inaperturate pollen was interpreted as a loss-of-function mutation of aperture formation, and an 8 bp deletion causes such male sterility in homozygous (FF) *Vitis* females [16,26]. This may have been an ancestral feature that was lost during the developing of hermaphroditism in the domesticated subspecies. New molecular data has recently demonstrated that dioecy has been lost during domestication through a rare recombination event between male and female haplotypes [26]. In some cultivars, and under particular conditions, the same character may return to be manifest as in the case of cv. Malbo Gentile. The nature of the new appearance as a plesiomorph character is still debated. It may be due to anomalies in the microsporogenesis process [8], or an abnormal division of the tapetum cells. Büyükkartal et al. [79] observed that the insufficient supply of nutrients to mother cells by the tapetum cells causes a low fertility of pollen in cv. Cavus. Any anomalous feature, then, may be fixed, possibly propagated by some anthropic practices like seedling [80].

4.2. The Current "Ancient Cultivars"

The cultivars analyzed in this research can be considered all ancient cultivars, according to agrarian and historical studies. Covra has been mentioned for the first time in 1752 by Niccolò Caula, as reported a century later by Maini [81]. Currently, this variety, which has a typical distribution in the Emilia region, has become rare due to the replacement with more productive varieties with larger clusters. This replacement is known to have occurred between the end of the 19th century and the beginning of the 20th century, and to the abandonment of viticulture in mountain areas where Covra was cultivated.

Lambrusco Grasparossa, which is currently the second grape variety in terms of cultivated area in the province of Modena, has been present at least from the end of the 18th century [82].

Bianca di Poviglio is the local name of a variety that genetic analyses have revealed to be Trebbiano Toscano, one of the oldest Italian grapes widely cultivated in Italy, whose origin, however ancient, is uncertain from both geographical and historical point of view: based on molecular studies, it is assumed to refer to the Medieval or even to the Roman periods [83]. So far, no historical documents have been found on the appearance of the variety in Poviglio, beyond the oral evidence on the long link with that territory.

Malbo Gentile, with its synonym Amabile di Genova, has been cited for the first time in 1805 by Filippo Re in a manuscript, and then in 1840 by Bertozzi as reported in [84].

The study of the floral biology and pollen traits of ancient cultivars can provide useful information for verifying the relationship between wild and cultivated grapevines. As previously reported, the presence of functionally female flowers, here represented by cv. Malbo Gentile, constitutes an ancestral characteristic that seems to derive from the wild vines. The comparison of pollen found in archaeological sites with that of cultivated varieties presenting ancestral characteristics suggests possible connections between the use of (wild) grapes and its domestication.

The first genetic evidence of the link between wild grapevine and varieties grown in the 19th century is now available and concerns the Emilia area. Analyses of molecular markers SSR and SNP on over 300 genotypes of cultivated varieties, rootstocks and wild vines give a first experimental evidence of the close relationship between wild vines and Lambrusco di Sorbara, another ancient cultivar with inaperturate pollen and male sterility, as well as with "Lambrusco a Foglia Frastagliata", a variety with true hermaphroditic flowers [85]. Today, the wild grapevine populations are an important resource for breeding programmes in order to avoid genetic erosion and in the perspective of a possible adaptation of the grapevines to the future environmental changes [86].

4.3. Viticulture during the Bronze Age in the Po Plain

Archaeobotany and organic residues in ceramic vessels allowed investigating early cultivation and use of *Vitis vinifera* in Georgia [42,87], the Near East [88] and the eastern Mediterranean [89]. The most ancient proofs of wine production may be dated to the Neolithic (6000–5800 BC) without the possibility of distinguishing if the wine was produced from wild or domesticated *Vitis vinifera* both exploited in the South Caucasus [42]. Even more ancient proofs (7000 BC) of production of a fermented beverage come from a local, high-sugar wild vine in China but it was a mixed wine–juices drink [90].

Starting from a quite familiar concept dealing with wild cereals—for which plant processing and cultivation is assumed to have begun long time before domestication (for example, see Mercuri et al. [91])—we assumed that the wild plants of *Vitis* should have been exploited for food or other uses for a long time. Even wild vine was known to be useful to prepare some fermented drinks, and therefore people had enough cultural knowledge assuring an easy transposition of practices from "old" to "new" plants producing more pleasant fruits. However, wild vines are thought to produce far less fruits than the domesticated vines [42], as male plants looked sterile to people. This should have involved a rapid adoption of the domesticated species, which demonstrated to be more suitable for food production [92], for example because all plants give fruits and there is more fruits production over the same surface.

In Italy, the cultivation of *Vitis vinifera* is traced back to the beginning of the Iron Age in relation to the presence of the first Greek colonies in southern Italy. However, remains of pips from the Bronze Age suggest that the beginning of its cultivation can be anticipated of several centuries in mainland Italy and Sardinia [47,93]; also pollen analysis suggests an early *Vitis* cultivation around 2000 BC in the Massaciuccoli Basin, Tuscany-central Italy [94].

An early consumption of products from this species, i.e., wine or vinegar or must, was suggested by Pecci et al. [52] based on organic residues analysis conducted on pottery from Canale Anfora-Aquileia, near Udine, and from Pilastri di Bondeno, near Ferrara. These two sites of north-eastern Italy dated to the Bronze Age. Pilastri di Bondeno is part of the Terramare culture. Moreover, seed/fruit remains found in the Terramara di Montale near Modena suggested that also other plants were used in the preparation of alcoholic drinks. *Cornus mas*-cornelian cherry has been among the most common and used plants to prepare alcoholic drinks since the beginning of the Terramare culture. Then, the exploitation of grapevine increased at the transition between the Middle Bronze Age (ca. 3600–3450 years ago) and the Late Bronze Age (ca. 3450–3170 years ago [95,96]) testifying to a preference for grapevine over the cornelian-cherry. Cornelian cherries continued to be used and were probably processed in the Etruscan farmhouse of Pian d'Alma, southern Tuscany, during the 6th century BC [97].

Therefore, the archaeobotanical context of the Terramare suggests that *Vitis vinifera* was known and used in the Po Plain about 3600–3100 years ago [96]. This alluvial plain was full of wet habitats suitable for the wild vine subspecies. It probably lived in hygrophilous woods near rivers, while the oak woods marked the limits between the villages. The use of grapevine was probably favored by a combination of local environmental conditions and cultural knowledge. In fact, trizonocolporate *Vitis* pollen is common in the sites of the Po Plain as a direct evidence of plants living close to the sites or in the surrounding areas.

As mentioned above, in the Terramara S. Rosa di Poviglio, the high percentage of *Vitis* pollen (18% in sample P19) clustered with riparian and willow communities thus suggesting the wild vine grew near ditches and channels of the site [60]. The interdisciplinary research demonstrated that people had a sophisticated knowledge of the land, also being able to shape the water and wood landscapes for improving their stability and resources exploitation [58,61]. We concluded that also the records from *Vitis* pollen may be actually a further evidence of some management of the plant at the Middle and Recent Bronze Age phases, and that grapevine was cultivated, even not domesticated, in the site [60]. The inaperturate pollen supports this interpretation adding the information that these plants also produced pollen from female flowers.

4.4. Inaperturate Pollen as Evidence of Wild Plants in Past Records

The reference pollen collected from the living plants studied in this research corroborates the assumption that the inaperturate pollen is a feature proper of *Vitis vinifera*. However, our observations confirm that the lack of colpora brings serious problems of fertility. This is irrelevant in the wild dioecious subspecies; the presence of remnants of hermaphroditism does not reduce the functionality of female flowers with anomalous stamens (Figure 1), and the inaperturate pollen only acts as a reward for pollinators.

Sterility may be a major handicap for vineyard yield and, therefore, inaperturate pollen is a rare and not favored character in many cultivars of the domesticated subspecies. Actually, the ancient cultivar Malbo Gentile produces only this type of pollen, seriously carrying fall of productivity if it is cultivated alone or far from hermaphroditic grapevines serving as pollinizer [29]. This anomaly occurs also in other cultivars such as Lambrusco di Sorbara, Picolit, Trebbianina (Trebbiano di Spagna), all registered in the "national catalogue" of grape varieties, and other local minor grapevines [27,31].

Today, the presence of inaperturate pollen is a not common trait in modern cultivars. Moving back to the Bronze Age in Italy, the general context and the whole pollen data obtained from the Terramara di Poviglio (see the inclusion of *Vitis* pollen in the hygrophilous wood group based on the figure 11 in [60]) strongly suggest that the inaperturate pollen was produced by wild dioecious plants of *Vitis vinifera* subsp. *sylvestris*.

In general, the inaperturate *Vitis* pollen in archaeological samples is an index of the presence of female flowers. In prehistoric times and in appropriate contexts, the compresence of the two types of *Vitis* pollen may be a good and reliable index of the presence of wild, or intermediate forms, of grapevines. This is of crucial importance dealing with a period of transition between the use of wild and domesticated grapevine.

5. Conclusions

Pollen dimorphism demonstrates that pollen production and dispersal differ in the wild and domesticated subspecies of *Vitis*, and therefore the signal of *Vitis* pollen in past records changed depending on which subspecies was present. This makes grapevine botanically different from the other Mediterranean cultural trees. In fact, *Olea*, *Juglans* and *Castanea*, which are included in the OJC index [98], did not change their reproductive strategies during domestication as grapevine, and consequently comparable pollen production and dispersal are expected in wild and domesticated plants. The proposal of an OJCV index (where V means *Vitis*) [99,100], which ignores this difference, seems not justified from a palynological point of view.

Moving to the basic question of this paper, can the inaperturate pollen be a marker of female wild *Vitis vinifera* in prehistoric times? Inaperturate pollen has never been detected so far in archaeological sediments. In the site studied here, Terramara Santa Rosa di Poviglio, we did not find such inaperturate pollen in layers dated to the more recent phases, subsequent to the Bronze Age, that surely refer to the cultivation of domesticated *Vitis* in the Po Plain. Therefore, we can conclude that our Bronze Age record bears witness of a very local growing of *Vitis vinifera*, and that the occurrence of dimorphism is coeval with the Bronze Age.

Based on our data, considering the inaperturate pollen a rare (plesiomorphic) trait in the domesticated grapevine, we suggest that the record should be interpreted according to the chronology and the archaeological context. Particularly, the record of both trizonocolporate and inaperturate pollen in the same samples may be a good indicator of wild grape—*Vitis vinifera* subsp. *sylvestris*—in prehistoric times.

Locally, the interdisciplinary research carried out in the Terramara Santa Rosa di Poviglio suggested that the (wild) plants grew in hygrophilous woods, next to the ditch and to the river, were cared for or cultivated during the Middle and Late Bronze Age periods [60]. In a general perspective, a complex agro–sylvo system and high skills in agriculture practices characterized the Terramare's people who were aware of the diversity of natural resources [61]. Grapevine processing and preparation of beverages—as wine or other products releasing tartaric acid in special vessels—were well known [52]. Nevertheless, we can agree with McGovern and colleagues [42] who suggest that the history of viticulture "illustrates human ingenuity in developing horticultural and winemaking techniques, such as domestication, propagation, selection of desirable traits, wine presses, suitable containers and closures, and so on". The plants growing next to the villages were wild grapevines, protected and managed by people who also knew how to exploit them, for food and wood. As hypothesized by Zohary [74] about selection criteria, Bronze Age people have selected plants producing fruits for centuries, and this has favored both female and the wild hermaphroditic grapevine growing in the same places. The latter wild plants were observed in Georgia, a region that is regarded as one of the earliest areas of *Vitis* domestication [20]. The case study of Poviglio further demonstrates that the technological competence should have been acquired before domestication, allowing the shift of the same practices (cultivation, processing) from the wild to the domesticated species, subspecies in the case of *Vitis vinifera*.

We conclude that the inaperturate pollen of *Vitis* found in the archaeological layers of the Terramara Santa Rosa di Poviglio testifies to the presence of wild grapevine close to the archaeological site. This is of special importance because Bronze Age is known to have been a period of transition between the grape collection in the wild and the introduction of the domesticated grape subspecies in the Italian peninsula [47,60,90] as well as in the East Mediterranean [53,101]. This study adds new important elements to the knowledge of the relationships between people and grapevine, also underlining the importance of botany in understanding environmental contexts and archaeology, crop history, and human cultural evolution.

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