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PHYSIOLOGICAL STUDY OF
VITIS VINIFERA L. SUBSP. *SYLVESTRIS*

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Questa ricerca scientifica, che mi ha consentito di ottenere il più alto titolo d'istruzione a oggi conseguibile in Italia, la dedico a:

MARCO, SILVANA, TILDE, MELANIA e VITO.

TABLE OF CONTENTS

ABSTRACT

The wild grapevine, *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi, is a very ancient plant. For this reason, its presence in a determined area is symbol of a millennial tradition of culture of grapevine. Here is why in the actual conception of the wine as expression of a territory, the autochthonous or "local" vines constitute an element of strong identity. They are the custodians and the vehicle of a local authentic cultural property so it is very important safeguard of the autochthonous vines and their biodiversity. The preservation of wild populations of *V. vinifera* L. subsp. *sylvestris* is essential for the maintenance of genetic variability and to resist at the genetic erosion. The intensive cultivation of the grapevine in extensive areas using only a few varieties and clones, has drastically decreased genetic variability and has increased the risk of an epidemic disease. The future of *Vitis vinifera* L. subsp. *sylvestris* represents a major stake in biodiversity conservation. Italy, among the other European wine countries, is one of the most rich of diversification in cultivar varieties and this is a strong advantage for the typical production of both grapevine and wine. Moreover, some area in our country, such as Sardinia and Tuscany, are very rich of local wild vines. This last represents an exaltation of biodiversity, not only as a biological difference, but also as a cultural product of the population's history. Aware about the scientific importance of these plants, this work was initiated for the safeguard and the ampelographyc, molecular, pathological and phenological study of wild vines. More than one hundred and forty accessions of *Vitis vinifera* L. subsp. *sylvestris* were recovered, on several sites of the Tuscany "Maremma" and classified, of which 76 were planted in a collection vineyard and trained to a special trellis system (Totem) to be observed in the same environmental conditions. Female plants having mainly berries of black colour were prevalent respect to male vines. The characterization consisted on: polymorphism of microsatellites loci (SSR); ampelographyc (OIV, 2009) and ampelometric (by computer assisted method "Superampelo") assessment; pathological monitoring (9 virus tested: GFLV, ArMV, GLRaV-1,-2,-3,-7, GVA, GVB, GFkV, and fungal infection incidence of *Plasmopara viticola*); thermal requirement for bud breaking (in growth chamber and phenological study in situ); monitoring of technological grape ripening; micro-vinification and chemical analysis of the wines obtained; secondary metabolites, polyphenols richness and anthocyanins profiles. Data were subjected to multifactorial analysis with standardization where necessary. The nuclear microsatellite profiles showed a wide diversity between the accession tested regardless to the area of origin.

Moreover certain supposed accessions of *Vitis vinifera* subsp. *sylvestris* retrieved seem to derive from the vines already cultivated that become wild, while others accessions would be intra-specific cross-breeding *sativa-sylvestris*. Only 6 accessions showed mainly single virus infections, or in association. Slight differences on fungal infections susceptibility were also found between the accessions tested, while several of them were less susceptible than cv. Sangiovese. Ampelographyc traits of shoot, leaf, grape and berry and ampelometric observations allowed to distinguish the different accessions, which showed a good similarity within the same area of origin. The material retrieved, other than to have a large morphological and genetic variability, evidenced very particular anthocyanins profiles which were different from the most common grape variety cultivated in Tuscany. In addition, the accessions studied had small clusters and berries with a satisfying ripening state and a rich polyphenol content. Also the wines obtained by micro-vinification of several biotypes, subjected to chemical analysis had evidenced differences suggesting possibility of enhance. Several accessions of *V. v. sylvestris* are able to ripe grapes of acceptable quality, with a reduced use of pesticides. In some cases giving a wine fairly acceptable that can be improved (if not used directly) or it could help to identify varieties with different levels of diseases's susceptibility. Lastly, the study of the main characteristics could be useful to find out some favourable traits to make a further genetic improvement of our varieties.

1. INTRODUCTION

1.1 Historical notes

The history of the grapevine, *Vitis vinifera* L., is enormously long. In particular, it is much old, and date back to around 100 million years ago. The first *Vitaceae* fossils date back to the Cretaceous (more than 165 million years ago), while the genera *Vitis* had the highest diversity during the Miocene (20 million years ago). During that period, had also lived the *Vitis praevinifera* L., the ancestral species from which they are made to derive a large part of the current ones. However, only one specie was really made domestic (cultivated), while the others remained practically wild.

The primitive forms of *Vitis*, were hermaphrodites, similar to the current ones. Also during the phylogenetic evolution, the vine have almost always shown hermaphrodites genetic traits (stamen plus anthers which contain the pollen, and the pistil with the ovary, for the development of the seed on the same flower). The advantages of this evolutionary line, which facilitates the reproduction, are obvious: first of all the capacity of auto-fecundation.

But, during the Quaternary glaciations (2 million years ago), due to the hard climatic conditions, the grapevine became dioicus in all the areas of origin (Munoz-Organero *et al.*, 1999). So, separate sexes were found on different plants. Everyone still had in the flower stamens and pistils, but in males, the maturation of one gene (SuF) of 38 chromosomes of the genetic kit, has suppressed the development of the female organ, and in females, the gene (SuM) started a recessive maturation, prevented the development of male stamen (Zdunic *et al.*, 2013). Only less of 5% of these plants have maintained their hermaphroditic character even after the glaciations.

According to the most reliable opinions, during the ice ages, some species of the genera *Vitis* survived in three different areas (Grassi *et al.*, 2003): one in North America, the second in East Asia, and the most important, the third, in Southern Europe (especially in the Caucasus and in Italy). In this latter area, survived the vine named European (*V. vinifera*), from which are derived all the current cultivated varieties, that are considered to belong to three different groups (Negrul, 1938; Cunha *et al.*, 2007):

1) proles occidentalis, with small berries, cultivated in western Europe and used for winemaking;

2) proles orientalis, with large berries for ready consumption, cultivated in Asia (table grapes);

3) proles ponticas, from Asia minor and eastern Europe, which presenting intermediate characteristics.

1.2 The origin of viticulture

Cultivation and domestication of grapevine appear to have occurred between the seventh and the fourth millennium B.C. in southern Caucasia, more specifically, in a geographical area between the Black Sea and the Caspian Sea (Zohary, 1995). That area may have constituted the primary domestication centre (Grassi *et al*., 2003; Arroyo-Garcia *et al*., 2006; Bacilieri *et al.*, 2013). In fact from that zone, cultivated forms would been spread by humans in the Near East, Middle East and Central Europe (Fig. 1).
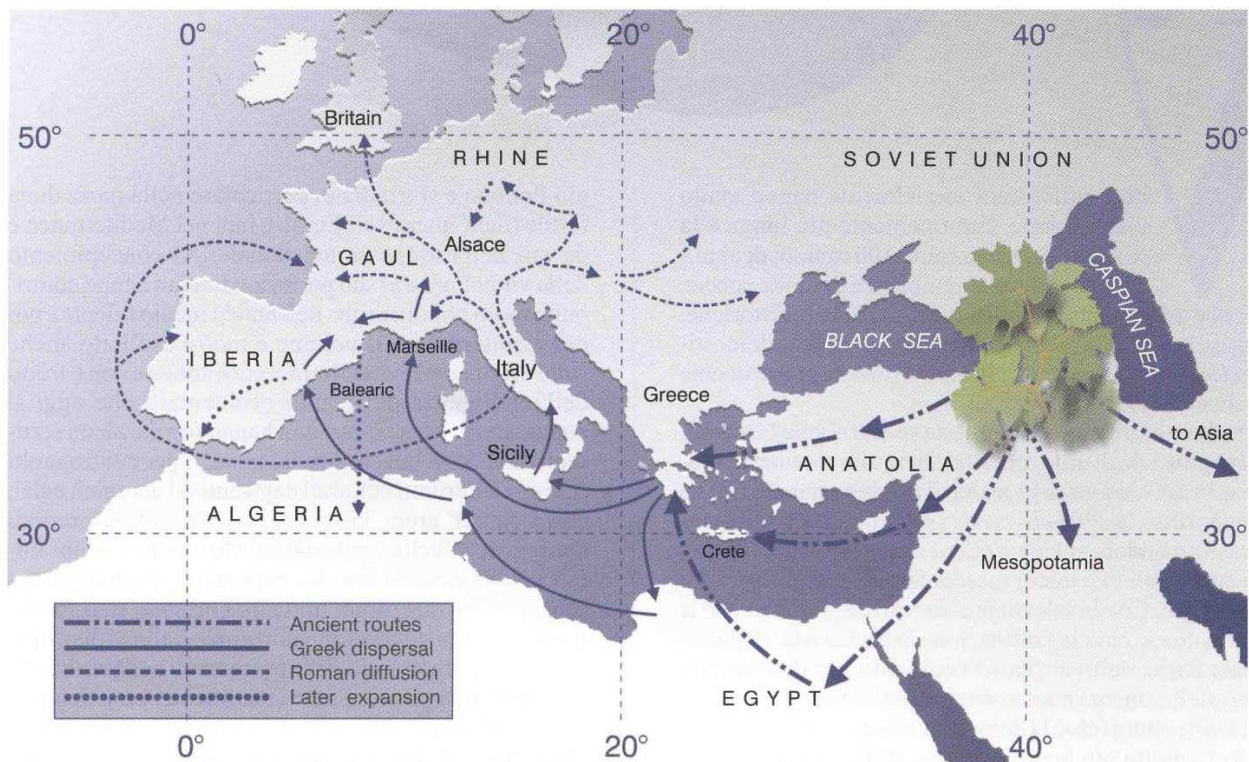
Figure 1: Main paths of viticulture, from Caucasus to Europe (Fregoni, 2013).

Primarily, were the Caucasian peoples and then Mesopotamian, Egyptians and Jews, that with the selection of the best plants, started the viticulture, which is much more short that the history of the plant, only about 10 millennia (Fregoni, 2013).

The domestication process involved the selection of that small part of hermaphrodite genotypes, which produce larger and sweeter berries of attractive colours, and the development of techniques for their vegetative propagation (Zohary, 1995). Then, grapevines were cultivated in Greece since the second millennium B.C., and thanks to Phoenician and Greek colonization, the vine was exported to other European regions, followed by the spread of viticultural knowledge.

After that, Romans became ambassadors of viticulture, and in association with the conversion to Christian faith, to drink wine became a part of European culture.

Major questions regarding grapevine domestication, concern the number of domestication events and the geographic locations where they took place.

It is commonly believed that the spread of viticulture also involved the dissemination of domesticated varieties of grapevines, while the use of indigenous wild vines is discussed as alternative origin of grapevine cultivars in central and western Europe.

For these reasons, two basic different hypotheses can be formulated:

1) a restricted origin hypothesis, according to which the domestication took place from a limited wild stock in a single location. These cultivars were subsequently transplanted into other regions (Olmo, 1995);

2) a multiple-origin hypothesis, according to which the domestication could have involved a large number of founders recruited, during an extended time period and along the entire distribution range of the wild progenitor species.

In agreement with the first possibility, archaeological research have traced the earliest evidence of large-scale's winemaking, presumably exploiting a domesticated plant. This discovery date back to the Neolithic period, carried out in the northern mountainous regions of the Near East, encompassing the northern Zagros, eastern Taurus and Caucasus Mountains (Zohary, 1995).

On the other hand, the existence of morphological differentiation among cultivars from distinct geographical areas in the Near East and in the western Mediterranean region, supports the second possibility, according to which, wild local *V. v. sylvestris* germplasm significantly contributed to the generation of grape cultivars, possibly through multiple domestication events (Levadoux, 1956).

Furthermore, this possibility is compatible with an eastern ancestral origin for the wine culture and viticulture practices and its spread from east to west.

Most authors do not particularly support either one or the other of these hypotheses, but emphasize that their relative importance for the development of European viticulture remains uncertain.

For example, Olmo (1995) explicitly doubts the role of Europe's scattered wild vine populations in domestication.

To resolve this issue has important implications to understand the origin of the current grape cultivars and provides information on the processes involved in the domestication of woody plant species.

Certainly, the analysis of the amount and the distribution of genetic variation in cultivated (*V. vinifera* subsp. *sativa*) and wild (*V. vinifera* subsp. *sylvestris*) populations can also help.

But, the introduction of many varieties by Greeks and Phoenicians to the western Europe and the intensive exchange of cultivars among vine-growing regions would be equivalent to a high rate of migration.

Therefore, as this type of migration was mediated by human transport, such as seafaring across the Mediterranean Sea, geographical distances would have been a minor influence on the rate of plant exchange. For this, differentiation among cultivars would have been blurred by high rates of migration, and genetic distances would not necessarily correspond to geographical patterns.

Anyhow, wild vines (*Vitis vinifera* subsp. *sylvestris*) were abundant in their indigenous range in Europe until the mid-nineteenth century, when the arrival of foreign pests, such as phylloxera (*Daktulosphaira vitifoliae*), and the destruction of their habitats, caused by urbanization and agriculture's intensification, bringing European wild vines close to extinction.

However, the greater number of varieties cultivated in Europe, and namely in Italy, could be originated from crosses between hermaphrodite varieties and wild native plants, and actually, they are of Asian origin. Moreover, vines found in their natural habitats, today are considered to be a mixture of pure *V. vinifera* subsp. *sylvestris* vines, cultivated grapevines (*V. vinifera* subsp. *sativa*), and sometimes also rootstocks (hybrids of North American species) that escaped from cultivation, and of crosses between these species (Arroyo-Garcìa *et al.*, 2006).

The combined action of selection, breeding, admixture and migration is believed to have shaped the cultivated compartment, possibly starting from multiple gene pools during domestication. Humans certainly selected traits related to fertility, blossom drop, productivity, berry size, sugar and acidity content, since these are keys for successful grape production. Similarly, hermaphroditism has been strongly selected for, almost to complete fixation, as self-pollinating plants achieve higher fruit production. Other traits were also probably selected, such as shoot habit, tolerance to biotic and abiotic stress, adaptation to local environment, and cuttings ability. Vegetative propagation is indeed believed to have been adopted early in the domestication process (Bacilieri *et al.,* 2013).

However, the domestication process remains largely unknown. Crucial unanswered questions concern the duration of the process (rapid or slow) and the related geographical area (single or multiple-origins). Seeds from domesticated grapevine and from its wild ancestor are reported to differ according to shape (Bouby *et al.,* 2013).

V. vinifera plants are highly heterozygous and the vegetative propagation of cultivars has maintained their high heterozygosis levels. When cultivars from the same geographic regions are grouped, nuclear DNA microsatellite markers provide weak discrimination between different geographic groups, with the greatest variation existing within the cultivar groups themselves.

Additionally, European grape cultivars have a complex history of movement over growing regions, which hampers the recognition of clear geographic trends in their distribution (Sefc *et al*., 2003).

Finally, viticulture represents a real culture. The ancient history is very useful to understand the modern, which is only a century old.

The world vineyards had almost reached 10 million hectares in 1960-1970 and in 2011 they amounted to 7.585.000 hectares.

1.3 Classification

The grapevine belong to the order of *Rhamnales*, family *Vitaceae* or *Ampelidaceae* which is divided into two subfamilies: *Lecoidaceae* and *Ampelideae*. To the latter belongs the genera *Vitis* and other four genera used for ornamental purposes.

In the genera *Vitis* we find about 40 Asiatic species plus other 30 American, belonging to two subgenera: *Muscadinia* and *Vitis* (Fig. 2). All these species are generally inter-fertile, and diploid (chromosomal patrimony 2n = 38), with the exception of the subgenera *Muscadinia*, which includes *Vitis rotundifolia*, with 2n = 40.

Among the group of European-Asiatic vines, typical of the temperate climates, we find the *Vitis vinifera*, which includes 2 subspecies: *V. v. sylvestris,* such as wild vines and *V. v. sativa,* such as the cultivated vines.
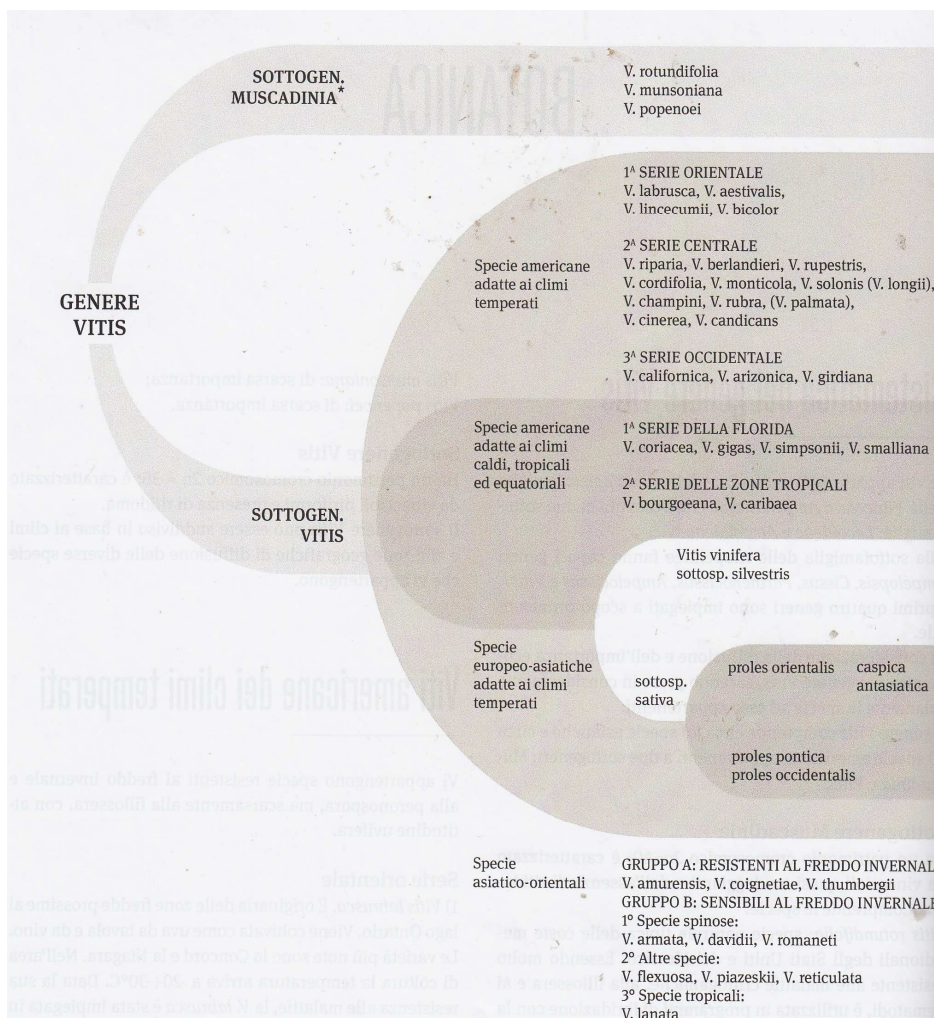
Figure 2: Classification of the genera *Vitis* (Fregoni, 2013).

A great majority of cultivars, now widely cultivated for fruit, juice and mainly for wine, classified as *Vitis vinifera* L. subsp. *vinifera* (or *sativa*), derive from wild forms [*Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi] (Sefc *et al.*, 2003; This *et al.*, 2006).

Serious discrepancies exist between different authors regarding the correct taxonomy of wild vines. While some consider *V. v. vinifera* L. and *V. v. sylvestris* (Gmelin) to be separate, others believe that the differentiation goes no further than the level of subspecies. According to the latter, these wild vines do not constitute a distinct species, nor even a subspecies, but rather are a group of genotypes within *Vitis vinifera* L. which develop in the wild state.

For the purposes of studying wild vines, the definitions and classifications proposed by Levadoux (l956) are very useful. According to this classification, wild vines may be of three types:

1) post-cultivated: deriving directly from a cultivated vine which was later abandoned;

2) sub-spontaneous: which appear in soil that was not cultivated but are derived from seeds coming from a cultivated plot;

3) spontaneous: which are a natural part of the flora.

This last category is then further divided into three groups:

a) the colonials (those derived from sub-spontaneous wild vines which find propitious conditions in their surroundings for growth under wild conditions);

b) the autochthonous (those deriving from ancestors which have never been cultivated);

c) the hybrids (those deriving from the hybridization of the autochthonous wild vines with any of the other forms).

Of all these wild vine types, only the autochthonous ones are authentic *V. v. sylvestris* vines, while the other types correspond to *sativa-sylvestris* crosses.

However, it is possible to differentiate between the three types of spontaneous wild vines by molecular DNA markers.

The different investigators who have studied wild vines all over the world refer to them using the denomination *V. v. sylvestris,* but without trying to discern the different types given above.

1.4 The wild grapevine

The *V. v. sylvestris* is wild, dioecious and spontaneous in Europa (Tuscany Maremma, Sardinia, Basque areas, Greece, etc…) for thousands of years. From the female plants are harvest grapes, usually black, to make a wine which is tannic and acid, but that after a long period of aging, becomes good and is said "lambruschino".

This plant was called in this way by Karl Christian Gmelin (German biologist, botanist and entomologist that, in 1788 published a new edition of "Systema Naturae" of Linneo, enriching it with many additions and modifications).

The wild grapevine is also an heliophilous liana, that differently to cultivated vines, growing generally along the river banks, and in alluvial or colluvial deciduous and semi-deciduous forest (Levadoux, 1956; Arnold *et al.,* 1998).

In particular, *V. v. sylvestris* is a very hardy specie although it prefers sunny and fresh areas, neutral or calcareous soils, and in these environments is combined with high Mediterranean vegetations and holm-oak woods, *Quercus, Populus* and *Fraxinus* subspecies' plants. It is a woody plant and climbs up trees, walls, ruins, bridges, etc… Moreover, it develops up to 20 m in hight, but it is creeping when it not finds support to climb.

The trunk, with a diameter up to 40 cm, often surrounds the plant on which it climbs. The wild grapevine is a long-lived species that can even exceed 300 years.

It has a rough dark gray cortex with longitudinal slivers that tend to break away, long brown or reddish color branches, and it is a deciduous plant. The leaves of male plants are deeply lobed, while those of female plants are entire or slightly lobed.

It blooms in May-June, has black spherical or oblong berries of about 5-7 mm of diameter.

It is distributed in a wide area from Western Europe to the Trans-Caucasian zone and around the Mediterranean Basin, except the most southern infra-Mediterranean and non-Mediterranean zones (Fig. 3). (Arnold *et al*., 1998).
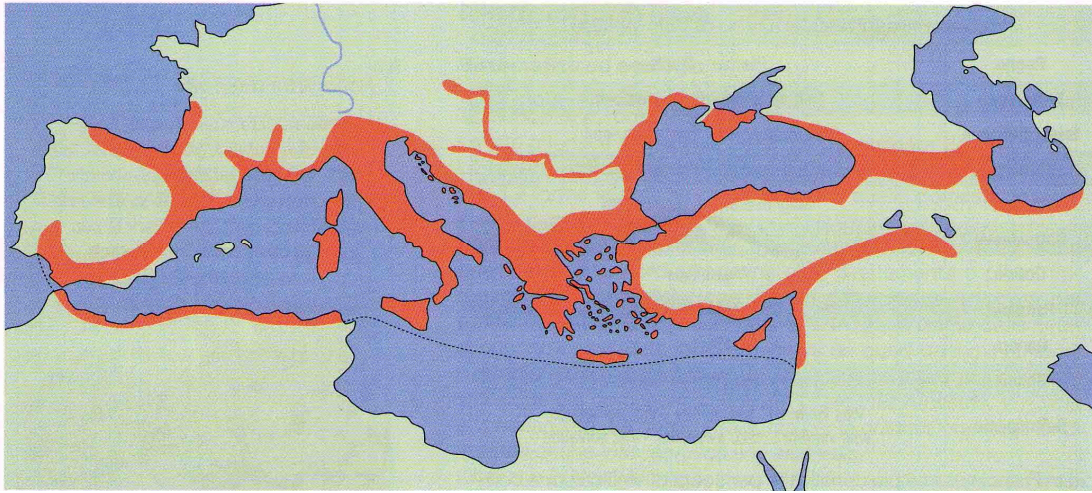
Figure 3: Area of *Vitis vinifera sylvestris*' distribution (Forni *et al.,* 2013).

Among these, the Iberian peninsula and Italy, in particular Sardinia (De Mattia *et al.*, 2007; Zecca *et al.*, 2010) and parts of Tuscany Maremma, seem to have the highest number of populations recorded.

The Nuragic civilization has developed in Sardinia since the second millennium B.C. Ancient Sardinians were an important part of the network of trade and cultural exchanges with other civilizations in the Mediterranean. The presence of remains of *Vitis* subsp. coming from Nuragic sites is documented in several excavations carried out in the island (Campus *et al.,* 2014; Orrù *et al.,* 2014).

Due to the ancient tradition of viticulture in Tuscany, grapevine cultivars (both autochthonous and non-autochthonous) are very numerous (Di Vecchi *et al.,* 2006).

It could be justified if we consider the two regions as "refuge areas" in the last ice age. This theory is confirmed by the fact that both populations of *V. v. sylvestris* in these areas have a higher level of genetic variability than other (Levadoux 1956; Grassi *et al.*, 2003).

However, the present distribution of the wild grapevine is highly fragmented in disjoint micro-populations or meta-populations, with few individuals, at least in the western part of the Mediterranean Basin.

This taxon is seriously endangered by human activities such as forest cleaning and setting fires. Moreover, invasive *Vitaceae* of the North American origin, imported after phylloxera (*Daktulosphaira vitifoliae*) when vineyards were being replanted, increase the risk to lose these spontaneous vines (Ocete *et al.,* 2012).

1.5 Differences between wild and cultivated

The vine is a very old plant and highly heterozygous. Propagation by seed carried out in the past, has also led to the creation of a large number of genotypes that have spread over a wide range of soil and climatic environments accumulating numerous mutations and thus increasing the genetic variability of populations.

To this we must add the great adaptability of the plant that changes its appearance depending on the environmental conditions.

At the genetic level as well as from the ampelographyc point of view, the two subspecies of *V. vinifera* are related but divergent (Zecca *et al.,* 2010).

So, it is very important underline the differences, at least the most important, that allow us to distinguish cultivated vine (with its many varieties and diversity, even clonal), from wild, also them very different among themselves. Therefore, the principal distinctions are (Fig. 4):

1) the existence of male and separate female plants in wild populations, although the flowers are morphologically hermaphrodite. In fact, they are dioecious and they necessity of cross-fecundation. Even if the existence of very hermaphrodite plants is also possible, but these do not exceed 5% the total of the population (Anzani *et al.,* 1990). In contrast, cultivated varieties are hermaphrodite and they have the possibility of auto-fecundation (Scossiroli, 1988);

2) in wild populations the tip of the young shoot is always open;

3) the size of the grapes and the clusters are more smaller in wild than the cultivated and also the density of the bunches is very loose;

4) there are differences about the size and the shape of the seeds. In wild they are smaller and rounded with beak smaller than the transversal diameter;

5) among wild-vines there is a leaf dimorphism. The leaf of male plant are more incised with sinuous lobes, while in female plant they are whole, a bit lobed with short stem.
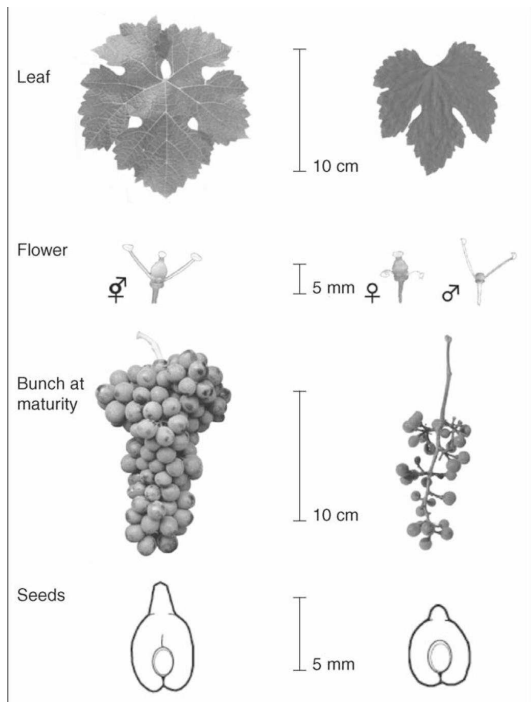
Figure 4: Mainly differences between wild and cultivated vine (Fregoni, 2013).

According to Levadoux, the most important characteristics for the differentiation of the different types of wild vines are the dioecious character, the opening of the petiolar sinus, and the shape of the seed. The size and the shape of *V. v. sylvestris*'s seeds are a determining and discriminatory factor by their very identification and, in particular the ratio of largeness/length (Levadoux 1956; Cunha *et al.,* 2007).

Besides those already mentioned, there are others minor morphological and physiological differences between wild and cultivated vines, in fact *V. v. sylvestris* has:

- high vigor, with trunk diameter up to 40 cm and height growth up to 10 meters (Ocete *et al.*, 2011);

- strong resistance to low temperatures;

- short growing season;

- different flowering's time (This *et al.,* 2006);

- phenological season of ripening and maturation often irregular (Ocete *et al.,* 2003);

- white varieties rather rare, moreover the berries are slightly juicy and with high acidity;

- variable pilosity of the leaves's lower page, from weak to medium (Levadoux, 1956; Arnold *et al.,* 2004).

The study of all these characteristics in existing wild vine populations, would be of great interest for the determination of the type of material to which they belong from Levadoux's classification.

1.6 Aim of the work

Aware about the scientific importance of these plants, this work was initiated for the safeguard and the ampelographyc, molecular, pathological and phenological study of wild vines, through the research, recovery, characterization and enhancement of different accessions of *Vitis vinifera* L. subsp. *sylvestris* mainly recovered in Tuscany "Maremma".

In particular, the aim of the project is the study of several factors until unknown that concern *Vitis vinifera* L. subsp. *sylvestris*, and the close examination to understand the phylogenetic relationship with *Vitis v. vinifera*.

More than one hundred and forty accessions of *Vitis vinifera* L. subsp. *sylvestris* were recovered, on several sites of the Tuscany "Maremma" and classified, of which 76 were planted in a collection vineyard. The characterization consisted on: molecular characterization of the DNA polymorphism (SSR); ampelographyc (OIV, 2009) and ampelometric (by computer assisted method "Superampelo") assessment; pathological monitoring (9 virus tested: GFLV, ArMV, GLRaV-1,-2,-3,-7, GVA, GVB, GFkV, and fungal infection incidence of *Plasmopara viticola*); thermal requirement for bud breaking, in growth chamber and phenological study in situ; monitoring of technological maturation of the grapes; micro-vinification and chemical analysis of the wines obtained; characterization of secondary metabolites, anthocyanins profiles and polyphenols richness.

A particular attention has been given to the study of cv. Sangiovese, considering the dominating role of this variety in the past and present history of the viticulture in Tuscany (Di Vecchi *et al.,* 2006).

The investigations could show a considerable variety of forms among of which, might be present biotypes of botanical or farming interests.

It can not finally to be overlooked as some varieties of *V. v. sylvestris* are able to ripe grapes of acceptable quality, with a reduced use of pesticides. In some cases giving a wine fairly respectable that can be improvable (if not used directly, however, it could help to identify varieties with different levels of disease's susceptibility).

Lastly, the study of the main characteristics could be useful to find out some favourable traits to make a further genetic improvement of our varieties.

2 MATERIALS AND METHODS

2.1 Plant material

The research was conducted in 3 consecutive years (2012, 2013 and 2014), principally on the adult plants in the collection vineyard of *V. v. sylvestris*, that were planted at "ColleMassari" farm (Cinigiano - GR) in the previous years.

It is composed of 76 different accessions (Tab. 1) coming from different habitat of the Maremma Toscana (Fig. 5).

Table 1: Accessions' list in the collection vineyard with their respective origins, sex, and berries' color.

	Accession	Origin	Sex	Color
1	Alberese Gr- 121/2	Alberese	M	-
2	Alberese O.F.-121	Alberese	F	B
3	Davanti cella	Alberese	F	B
4	25 parco	Alberese	F	?
5	36 parco	Alberese	F	?
6	35 parco	Alberese	?	?
7	18 parco	Alberese	?	?
8	23 parco	Alberese	F	?
9	24 parco	Alberese	?	?
10	16 parco	Alberese	?	?
11	7 prima su rovo	Alberese	?	?
12	9 roccia cavalleggeri	Alberese	F	B
13	Syl 109	Alberese	?	?
14	37 parco	Alberese	F	?
15	22 parco	Alberese	?	?
16	15 parco	Alberese	?	?
17	19 parco	Alberese	?	?
18	5 strada	Alberese	?	?
19	6 strada	Alberese	?	?
20	10 lato caverna	Alberese	F	B
21	8 albero cavalleggeri	Alberese	?	-
22	S. filippo 2	Campiglia d'Orcia	F	B
23	Capalbio 1	Capalbio	F	B
24	Morcola 5 olmo	Capalbio	F	B
25	Morcola 1 edera	Capalbio	F	B
26	Morcola 2 giov	Capalbio	F	W
27	Morcola 3 alloro	Capalbio	F	B
28	Morcola 4 prunus	Capalbio	M	-
29	Sforzesca syl 57	Castell'azzara	M	-
30	Lionero 4 rete syl 54	Manciano	F	B
31	Lionero 52	Manciano	F	W
32	Nera	Manciano	F	B
33	Quercia grande	Manciano	M	-
34	Querciola syl 43	Manciano	F	?

	Accession	Origin	Sex	Color
35	Siepone syl 45	Manciano	M	-
36	Syl 39	Manciano	F	B
37	Syl 41	Manciano	F	B
38	Syl 42	Manciano	F	?
39	Syl 53	Manciano	F	?
40	Syl 87	Manciano	M	-
41	3 Cantoni	Paganico	F	W
42	Ombrone 1 (b)	Paganico	F	W
43	Casa corto 1	Pian castagnaio	?	?
44	Alberello 96	Poggi del Sasso	M	-
45	capannelle tardivo syl 106	Poggi del Sasso	F	B
46	Cortilla	Poggi del Sasso	M	-
47	Cortilla lago	Poggi del Sasso	F	B
48	F. poggi	Poggi del Sasso	F	B
49	Maschio poggi	Poggi del Sasso	M	-
50	Mz bianco	Poggi del Sasso	F	W
51	Mz rossa	Poggi del Sasso	F	B
52	Mz 5	Poggi del Sasso	F	B
53	Nera 2F (sangiovese)	Poggi del Sasso	ERM	B
54	Mazzocchi 2	Poggi del Sasso	F	B
55	Syl 29	Poggi del Sasso	?	?
56	Biondi 1	Sorano	F	B
57	Biondi 2	Sorano	M	-
58	Biondi 3	Sorano	M	-
59	Biondi melo	Sorano	F	B
60	Biondi nera	Sorano	F	B
61	Del casco	Sorano	F	B
62	Cavone	Sorano	M	-
63	Piano 6	Sorano	F	B
64	Piano 7	Sorano	M	-
65	Poggio syl 76	Sorano	M	-
66	Segno 1	Sorano	F	B
67	Segno 2	Sorano	M	-
68	Montebuono F1	Sorano	F	B
69	Montebuono F2	Sorano	F	B
70	Montebuono F3	Sorano	F	B
71	Picciolana 77	Sorano	M	-
72	Syl 131	Sorano	?	?
73	Syl 78	Sorano	F	B
74	Syl 80	Sorano	M	-
75	Rocca silvana	Sorano	M	-
76	Grotte cavalieri	Sovana	F	B
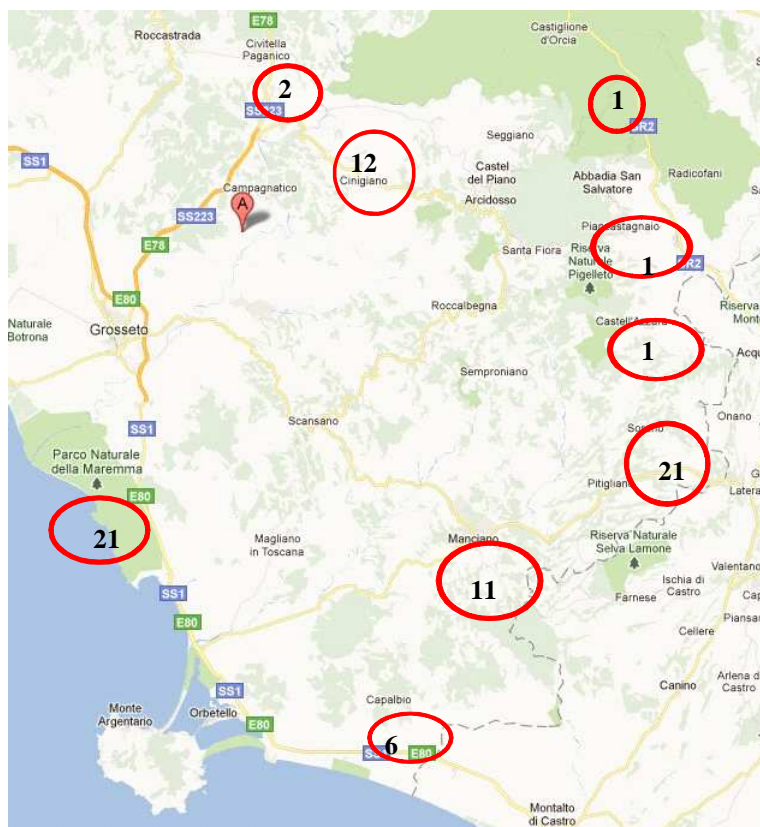
Figure 5: Areas of origin of the accessions in the collection vineyard.

The accessions with male flower (18) represent the 23.7 %, those female (42) are the 55.3 %, while (15) the 19.7 % are unknown (because they are very young plants), and one accession is hermaphroditic. In particular this last is a *Vitis v. vinifera* (cv. Sangiovese) coming from a nearly area, and it was intentionally planted in the collection to be considered as a reference in some tests.

Within the female the majority (29) are with black berry (69%), those with white berry (5) represents the 11.9 %, the rest part (8) are actually unknown.

The collection vineyard is composed of about 300 plants, planted without rootstock and with a system of cultivation named "Totem" (Fig. 6), which allows them a greater vegetative growth.

Figure 6: Training system adopted for the vineyard collection: Totem.

The characterization's process regarded almost always these accessions. In fact, few young accessions were not took in consideration, because they did not developed their vegetative organs in a better way, or they did not produce fruits. However, the analysis done, are:

- ampelographyc, according to the latest official methods (OIV, 2009);

- ampelometric, on adult leaves through the software "SuperAmpelo"

- pathological monitoring (free of virus and susceptibility to *plasmopara viticola* diseases);

- investigation on thermal requirement for bud breaking, in growth chamber and phenological study in situ;

- monitoring of technological maturation of the grapes and characterization of secondary metabolites: anthocyanins profile and polyphenols richness;

- chemical analysis of the wines obtained by micro-vinification;

- molecular characterization through an analysis of the polymorphism of microsatellites loci (SSR) as reported in D'Onofrio *et al.,* 2010.

Moreover, some searches in different habitats within the Maremma to discovered new accessions were done.

2.2 Ampelographyc and ampelometric characterization

Traditionally, the identification of grapevine variety and clones is obtained by "ampelography".

The word "ampelography" comes from the Greek words *ampelos* (grape) and *grafo* (describe) and literally means "description of grapevine". The actual ampelographyc methods are the following:

- descriptive methods, that describe the morphological characteristics of grapevine accession that allow to distinguish it from other accessions of another species, variety or clone;

- ampelometric method, that consist in the measure of some continuous organ parameters and they are less subjective that the ampelographyc methods.

The use of these methods in the variety and clone identification allow to obtain more accurate results.

In this work the ampelographyc and ampelometric characterization of *V. v. sylvestris* represents the central and most important part.

In the 1983, the O.I.V. (Office International de la Vigne et du Vin) published the "Code des caractéres descriptifs des variétés et espèces de Vitis" in that is reported the codification of ampelographyc descriptive characters, that allow their informatics management. In the O.I.V. tables, each character and their expression levels are identified by numeric codes. For the determination of the right level of expression of each characters some reference varieties have been indicated.

In this thesis the last edition of the O.I.V. method released on 2009, was adopted.

The ampelographyc characters are grouped in qualitative characters (with discrete expression levels), quantitative characters (with continuous expression levels) and alternative characters (presence, absence).

The ampelographyc characterization about the majority of the accession in the collection vineyard was made by visual reliefs in the field, every year (2012, 2013, 2014), because the different climatic conditions may be diversify the plant's organ. At the end, all data obtained in these 3 years were compared and for each character examined was extrapolated only one (or at most two) final datum that describes the accession for that character.

In addition, for those plants for which it were not possible the propagation at the moment of the discovering, some characterization were made in their natural habitat.

The observations involved the most important parts of the plant, such as: shoot at flowering, mature leaf, cluster, berry and seed.

In particular, for the shoot at flowering, we took in consideration the characters listed in the table 2. For the mature leaves, we characterized the accessions used the codes in the table 3. Finally in the table 4 are listed the codes used to characterize cluster, berry and seed.

Table 2: OIV code used for the ampelographyc characterization of shoot at flowering.

OIV code	Description	Notes
1	Young shoot: opening of the shoot tip	1 – closed
		3 - half open
		5 - fully open
2	Young Shoot: distribution of anthocyanin coloration on prostrate hairs of tip	1 – absent
		2 – piping
		3 – overall
3	Young Shoot: intensity of anthocyanin coloration on prostrate hairs of tip	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high
4	Young Shoot: density of prostrate hairs on tip	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high
5	Young Shoot: density of erect hairs on tip	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high
6	Shoot: attitude (before tying)	1 – erect
		3 - semi-erect
		5 – horizontal
		7 - semi-drooping
		9 – drooping
7	Shoot: color of dorsal side of internodes	1 – green
		2 - green and red
		3 – red
8	Shoot: color of ventral side of internodes	1 – green
		2 - green and red
		3 – red

9	Shoot: color of dorsal side of nodes	1 – green
		2 – green and red
		3 – red
10	Shoot: color of ventral side of nodes	1 – green
		2 - green and red
		3 – red
015-1	Shoot: area of the anthocyanin coloration on bud scales	1 – absent
		2 – basal
		3 – up to 3/4 of bud scale
		4 - almost on the whole bud scale
015-2	Shoot: intensity of anthocyanin coloration on bud scales	1 - none or very weak
		3 – weak
		5 – medium
		7 – strong
		9 - very strong
17	Shoot: length of tendrils	1 - very short
		3 – short
		5 – medium
		7 – long
		9 - very long
51	Young leaf: color of the upper side of blade (4th leaf)	1 – green
		2 – yellow
		3 – bronze
		4 - copper – reddish
53	Young leaf: density of prostrate hairs between main veins on lower side of blade (4th leaf)	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high
54	Young leaf: density of erect hairs between main veins on lower side of blade (4th leaf)	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high

55	Young leaf: density of prostrate hairs on main veins on lower side of blade (4th leaf)	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high
56	Young leaf: density of erect hairs on main veins on lower side of blade (4th leaf)	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high

Table 3: OIV code used for the ampelographyc characterization of mature leaves.

Oiv code	Description	Notes
65	Mature leaf: size of blade	1 - very small
		3 – small
		5 – medium
		7 – large
		9 - very large
67	Mature leaf: shape of blade	1 – cordate
		2 - wedge-shaped
		3 – pentagonal
		4 – circular
		5 - kidney-shaped
68	Mature leaf: number of lobes	1 - one (entire leaf)
		2 – three
		3 – five
		4 – seven
		5 - more than seven
70	Mature leaf: area of anthocyanin coloration of main veins on upper side of blade	1 – absent
		2 - only at the petiolar point
		3 – up to the 1st bifurcation
		4 - up to the 2nd bifurcation
		5 - beyond the 2nd bifurcation

71	Mature leaf: area of anthocyanin coloration of main veins on lower side of blade	1 – absent
		2 - only at the petiolar point
		3 – up to the 1st bifurcation
		4 - up to the 2nd bifurcation
		5 - beyond the 2nd bifurcation
72	Mature leaf: goffering of blade	1 - absent or very weak
		3 – weak
		5 – medium
		7 – strong
		9 - very strong
74	Mature leaf: profile of blade in cross section	1 – flat
		2 - V-shaped
		3 – involute
		4 – revolute
		5 – twisted
75	Mature leaf: blistering of upper side of blade	1 - absent or very weak
		3 – weak
		5 – medium
		7 – strong
		9 - very strong
76	Mature leaf: shape of teeth	1 - both sides concave
		2 - both sides straight
		3 - both sides convex
		4 - one side concave, one side convex
		5 - mixture between both sides straight and sides convex
77	Mature leaf: size of teeth in relation to blade size	1 - very small
		3 – small
		5 – medium
		7 – large
		9 - very large
78	Mature leaf: length of teeth compared with their width	1 - very short
		3 – short
		5 – medium
		7 – long
		9 - very long

79	Mature leaf: degree of opening / overlapping of petiole sinus	1 - very wide open
		3 – open
		5 – closed
		7 – overlapped
		9 - strongly overlapped
80	Mature leaf: shape of base of petiole sinus	1 - U-shaped
		2 - brace-shaped ({)
		3 - V-shaped
081-2	Mature leaf: petiole sinus base limited by veins	1 - not limited
		2 - on one side
		3 - on both sides
82	Mature leaf: degree of opening / overlapping of upper lateral sinus	1 – open
		2 – closed
		3 - slightly overlapped
		4 - strongly overlapped
		5 - absence of sinus
083-1	Mature leaf: shape of base of upper lateral sinuses	1 - U-shaped
		2 - brace-shaped ({)
		3 - V-shaped
84	Mature leaf: density of prostrate hairs between the main veins on lower side of blade	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high
85	Mature leaf: density of erect hairs between the main veins on lower side of	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high
86	Mature leaf: density of prostrate hairs on main veins on lower side of blade	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high

87	Mature leaf: density of erect hairs on main veins on lower side of blade	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high
93	Mature leaf: length of petiole compared to length of middle vein	1 - much shorter
		3 - slightly shorter
		5 – equal
		7 - slightly longer
		9 - much longer
94	Mature leaf: depth of upper lateral sinuses	1 - absent or very shallow
		3 – shallow
		5 – medium
		7 – deep
		9 - very deep

Table 4: OIV code used for the ampelographyc characterization of cluster, berry and seed.

Oiv code	Description	Notes
202	Bunch: length (peduncle excluded)	1 - very short
		3 – short
		5 – medium
		7 – long
		9 - very long
203	Bunch: width	1 - very narrow
		3 – narrow
		5 – medium
		7 – wide
		9 - very wide
204	Bunch: density	1 - very loose
		3 – loose
		5 – medium
		7 – dense
		9 - very dense

206	Bunch: length of peduncle of primary bunch	1 - very short
		3 – short
		5 – medium
		7 – long
		9 - very long
207	Bunch: lignification of peduncle	1 – only at the base
		5 – up to about the middle
		7 - more than the middle
208	Bunch: shape	1 – cylindrical
		2 – conical
		3 - funnel shake
209	Bunch: number of wings of the primary bunch	1 – absent
		2 - 1 - 2 wings
		3 - 3 - 4 wings
		4 - 5 - 6 wings
		5 - more than 6 wings
220	Berry: length	1 - very short
		3 – short
		5 – medium
		7 – long
		9 - very long
221	Berry: width	1 - very narrow
		3 – narrow
		5 – medium
		7 – wide
		9 - very wide
222	Berry: uniformity of size	1 - not uniform
		2 – uniform

223	Berry: shape	1 – obloid
		2 – globose
		3 - broad ellipsoid
		4 - narow ellipsoid
		5 – cylindric
		6 - obtuse ovoid
		7 – ovoid
		8 – obovoid
		9 - horn shake
		10 - finger shake
225	Berry: color of skin	1 - green yellow
		2 – rose
		3 – red
		4 – grey
		5 - dark red violet
		6 - blue black
226	Berry: uniformity of color of skin	1 - not uniform
		2 – uniform
227	Berry: bloom	1 - none or very low
		3 – low
		5 – medium
		7 – high
		9 - very high
228	Berry: thickness of skin	1 - very thin
		3 – thin
		5 – medium
		7 – thick
		9 - very thick
229	Berry: hilum	1 - little visible
		2 – visible
231	Berry: intensity of flesh anthocyanin coloration	1 - none or very weak
		3 – weak
		5 – medium
		7 – strong
		9 - very strong

232	Berry: juiciness of flesh	1 - slightly juicy
		2 - medium juicy
		3 - very juicy
233	Berry: must yield	3 – little
		5 – medium
		7 – high
235	Berry: firmness of flesh	1 – soft
		2 - slightly firm
		3 - very firm
236	Berry: particularity of flavor	1 – none
		2 – muscat
		3 – foxy
		4 – herbaceous
		5 - other flavor than muscat, foxy or herbaceous
238	Berry: length of pedicel	1 - very short
		3 – short
		5 – medium
		7 – long
		9 - very long
240	Berry: ease of detachment from pedicel	1 - very easy
		2 – easy
		3 – difficult
241	Berry: formation of seeds	1 – none
		2 – rudimentary
		3 – complete
242	Berry: length of seeds	1 - very short
		3 – short
		5 – medium
		7 – long
		9 - very long
243	Berry: weight of seeds	1 - very low
		3 – low
		5 – medium
		7 – high
		9 - very high
244	Berry: transversal ridges on dorsal side of seeds	1 – absent
		9 – present

304	Time of physiological stage of full maturity of the berry	
502	Bunch: weight of a single bunch (g)	
503	Berry: single berry weight (g)	
504	Yield per m^2 (Kg)	
505	Sugar content of must (°Brix)	
506	Total acid content of must (g/L)	
508	Must specific pH	

Furthermore, the ampelometric methods introduced the biometry in the study of continuous ampelographyc characters. The ampelometric methods are used essentially for two reasons:

- to obtain less subjective expression levels than ampelographyc characteristics;

- to obtain continuous parameters characteristic of some accessions and to have the possibility of to analyze these measurement with powerful statistical methods.

From the beginning of this characterization, the leaf appeared as the most appropriate organs for biometric studies, because they could be collected from the plants for a long period, are weightless, occupied few space, could be easily transported and finally they could be easily preserved after dried. Another advantage of leaves in biometric studies is their only two dimensions (their thickness is insignificant).

For this reason, few years ago was developed a software that allow us to calculate the measurable characters in a way most fast and simple, in order to have also the data in digital format.

This software named "SUPERAMPELO" (Soldavini *et al.,* 2013). It permit also to store in a worksheet a lot of numbers referred to the most important parameter of the leaves. Afterwards all these data can be statistically elaborated to make a classification most accurate than the subjective visual observations. In fact, for the leaf analysis were considered 23 characters belonging to the OIV classification, while with "Superampelo" selecting 63 points along the perimeter and inside the bottom page of each leaf, were calculated 88 parameters related to lengths and angles. Furthermore the software calculates the leaf type (form and size) of a group of leaves examined, with the medium-size of the data and the standard deviations of all the other leaves. Moreover can be calculated the similarity of a group of leaves and the similarities degree between the leaves standard of different cycles.

For this reason I took a sample of 20 representative mature leaves from each accession, in the area between the 6th - 7th to the 12th node along the shoot.

Then, the bottom page of each leaf was digital scanned at 100-120 dpi, while for best leaf the scansion was made at 400 dpi and an high-resolution photo was also made. The final scanned images were used for the software.

These ampelometric data were joined with those ampelographyc to get a complete description of the accessions.

2.3 Pathological monitoring

V. v. sylvestris seems to show, respect to *V. v. sativa*, a lower susceptibility regard the most important leaves diseases, whether they are of fungal origin, bacteriological or due to animals, such as insects and mites. These evidences can be attributed both to extrinsic factors, such as the environment, and intrinsic such as the greater rustic nature that characterizes this subspecies (Levadoux, 1956).

The environment on which they grow is characterized by the presence of the aphid *Daktulospharia vitifoliae*, responsible of phylloxera disease, that was not yet observed.

The infections of downy mildew (*Plasmopara viticola*) are highlighted with the appearance of leaf symptoms like wildfire and have not been reported macroscopic symptoms of berries (white rot or brown rot) (Ocete *et al.,* 2007). Powdery mildew (*Uncinula necator*) symptoms are visible on the clusters, and in case of severe infections even with obvious necrotic areas on leaves. However, the damage is minor compared to those identified on *V. v. sativa* subspecies (Ocete *et al.,* 1999; Ocete *et al.,* 2008).

Referring to viral diseases, instead, only few studies were to now completed.

The pathological monitoring regarded 2 test made under the supervision of Dr. Materazzi.

These observations allowed us to verify the resistance of the majority of the accessions in the collection to the downy mildew (*Plasmopara viticola*) infections. In 2013 the collection vineyard was not treated with the usual anti-fungus product. At the end of the spring and also in summer, 20 representative leaves of each accessions (44) were checked with a visual survey using a pathometric scale (Fig. 7) as suggested by Dr. Materazzi (personal communication) on which is marked the level of the fungus attack according to the number and the dimension of the stains.

| **Pathometric scale and value of the classes used** |
| Class 0 = healthy leaf |
| Class 1 = leaf area < 1% |
| Class 2 = leaf area < 5% |
| Class 3 = leaf area < 12% |
| Class 4 = leaf area < 25% |
| Class 5 = leaf area < 50% |
| Class 6 = leaf area < 75% |
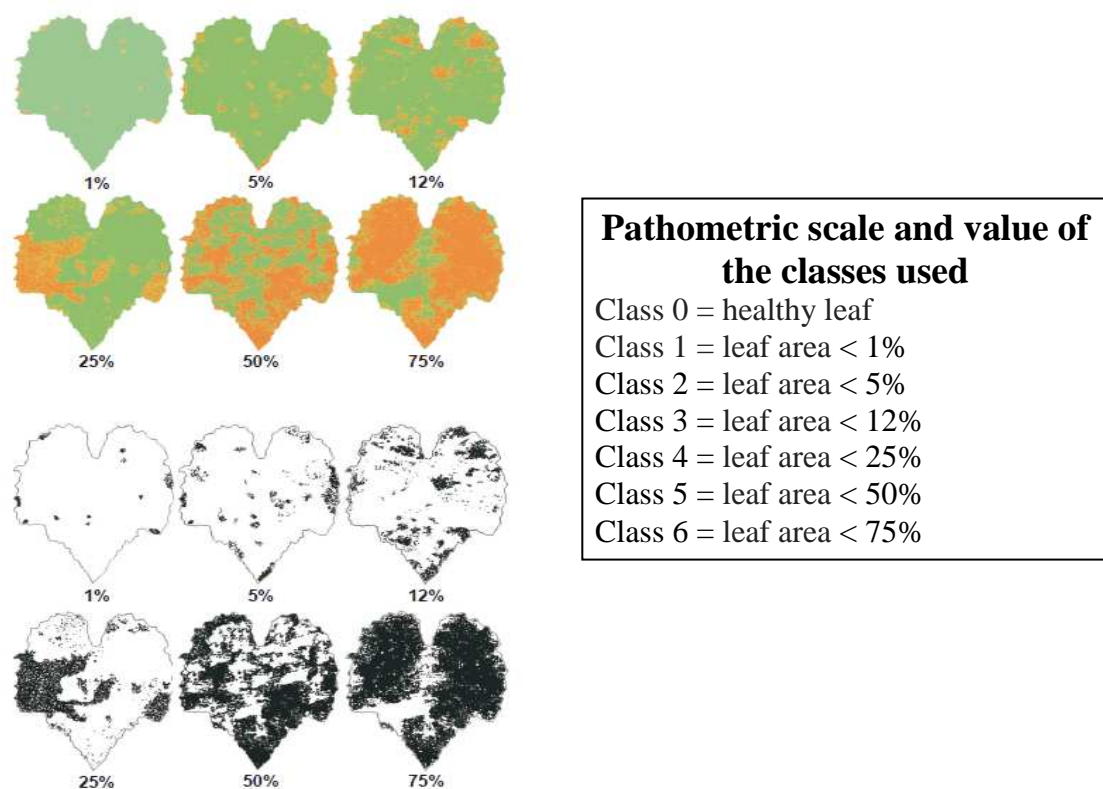
Fig: 7: Pathometric scale adopted.

Moreover, the same 44 accessions were further pathologically examined to monitor the possible presence of virus infections.

The phytovirological investigations were carried out using the ELISA test, to evaluate the possible presence of 9 different viruses: - Arabis mosaic nepovirus (ArMV); - Grapevine Fanleaf Nepovirus (GFLV); - Grapevine Fleck Maculavirus (GFkV); - Grapevine Leafroll associated Closteroviridae 1, 2, 3, 7 (GLRaV 1, 2, 3, 7); - Vitivirus A e B (GVA, GVB).

The samples analyzed were recovered during the winter season 2014. In particular, for all of the accessions investigated, were taken, in a random way, 5 portions of woody branches of the year of about 30 cm. Then the material was transferred at the Laboratory of Plant Virology in S. Piero a Grado (PI). Here was picked up and worked 1 g of phloematic tissue for each accession.

After that it was applied the ELISA (Enzyme Linked Immuno Sorben Assay) test, which is a very common virus-specific immunoassay method, both qualitative and quantitative. This technique is based on the combination of reactions, highly specific, between antigen and the corresponding antibody.

For the test was used the ELISA direct technique or DAS-ELISA (Double Antibody Sandwich) to verify the presence of ArMV, GFLV, GLRaV 1, 2, 3 and 7, while were respectively adopted, the variants "Protein A" and the "Direct Binding" for the determination of GVA and GVB. Finally, the indirect ELISA method or DASI-ELISA (Double Antibody Sandwich Indirect) was used for the identification of GFkV.

To make the phytovirological analysis of vine material with the ELISA method, there are appropriate kits consisting of the enzymatic reagents (buffers, monoclonal or polyclonal antibodies, positive and negative controls) specific to each entity viral investigated, commercially available.

Diagnostic procedures were conducted following the protocol of analysis provided by D.M./2011, which lays down the guidelines for the performance of phytosanitary analysis. The execution of the serological tests was used appropriate kits enzyme specific viruses.

2.4 Investigation on thermal requirements for bud-breaking

The *Vitis v.* subsp. *sylvestris* is characterized by a high genomic diversity, resulting from natural selection which established a strict relationship between the cultivar and the environment. Therefore, the accessions are characterized by a different physiological and morphological behaviour and the growing cycle depends on plant genotypes as well as on climatic conditions.

Dormancy is a physiological and physical state that allows a plant to survive periods of challenging environmental conditions such as low temperature in winter. As a woody perennial species, grapevine (*Vitis spp.*) enters dormancy in the fall following a genetic signalling cascade initiated reductions in photoperiod and decreases in temperatures (Londo and Johnson, 2014).

The aim of this test is to evaluate the morphological bud development during dormancy in field and to compare the heat requirement needed to start bud break in forcing condition, of six accessions of *V. v. sylvestris* also characterized by different geographic origins and one *V. v. sativa* (cv. Sangiovese) as reference (Tab. 5).

Table 5: Accession utilized for the investigation on thermal requirements for bud-breaking.

Accession	Orign	Sex
1	Alberese	M
26	Capalbio	F
28	Capalbio	M
48	Poggi del sasso	F
51	Poggi del sasso	F
53	Poggi del sasso	ERM
69	Sorano	F

Observations on bud development were made during the winter of 2013 and hourly temperatures were recorded by automatic data-loggers in the farm.

To determine the effective amounts of chill and heat, temperatures were transformed into Chill Units (CU) and Growing Degree Hours (GDH) according to Richardson method. (Andreini *et al.*, 2009). For the first, the method was based on the accumulation of the effective chilling hours during the winter season. One Chill Unit is equal to one hour of exposure below to 6.1°C and the chill contribution becomes less as the temperatures rise above or fall below this threshold. To complete the dormancy period, grapevines require relatively short exposure to chilling, ranging between 50 and 400 hours at temperatures < 7°C.

When the chilling requirement is satisfied, buds become sensitive to warm temperatures with the resumption of their active growth. This model is considered appropriate under the environmental conditions of Mediterranean areas.

The determination of CU began at the end of leaves fall while calculation of GDH started on 30th Julian Day (JD 1= 1st January). This was based on the assumption that early heat is not effective in promoting bud-break.

Based on the general BBCH-scale (Fig. 8), that describe the bud phenological stages, the accessions (30 buds for each one) were characterized in relation to the achievement of complete bud scale opening stage (03 of BBCH scale) which is suggested to consider as an early and indicator of bud-break in V*itis vinifera* (Andreini *et al.*, 2007), under the forcing and in field conditions.
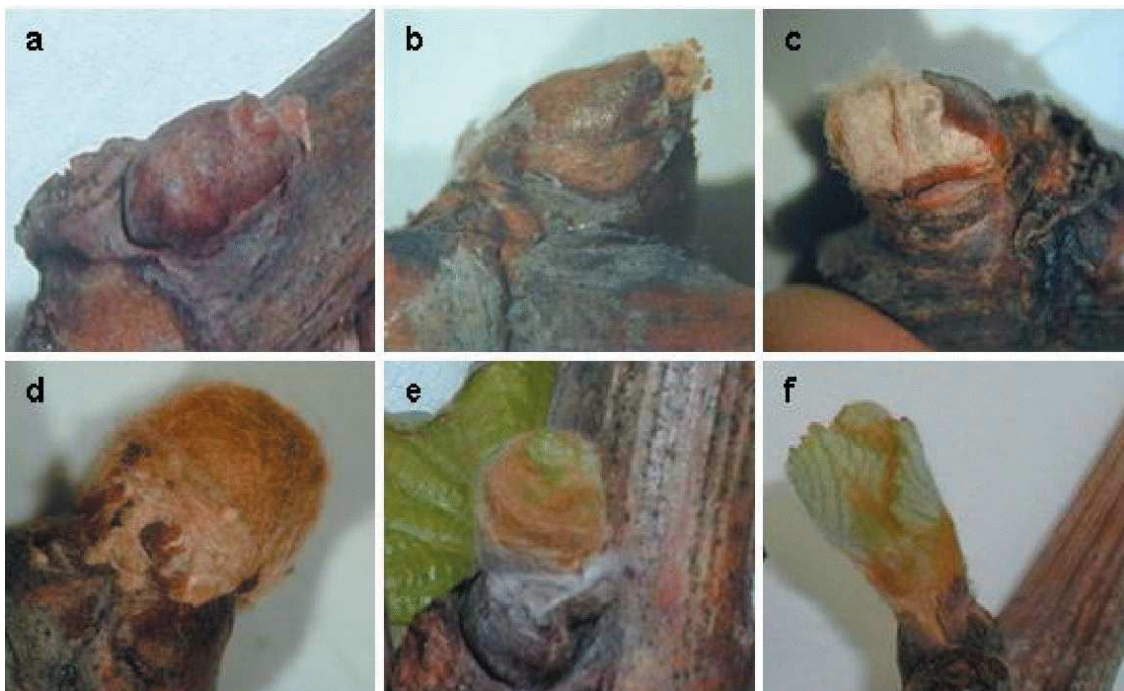
Figure 8: Phenological stages of bud evolution according BBCH-scale: 00 winter bud (a); 01 start of swelling (b); 03 bud scale opening (c); 05 woolly bud (d); 07 green tip (e); 09 bud opening (f).

The forcing test allow to evaluate the bud break in relation to the only genetic traits of each accession by removing the environmental factors (solar radiation, temperature variations, water availability and soil), which influence the process in field conditions.

In particular, the forcing conditions considered at 30 JD, cuttings (replication n= 3) containing 10 nodes for each accession. Thus, it was conducted on two-node segments maintained in water in a heat chamber at 25°C (±1) of temperature. The heat requirement for growth after rest was calculated using the following formula: GDH = 20°C x the number of hours during which the cuttings were forced, 20°C is the maximum efficacy temperature to stimulate bud-break.

Moreover, the bud's weight in field and after the forcing were evaluated, using samples of 30 buds for accession, and it was compared the middle stage of budding between 2012 and 2013.

2.5 Grape analysis, phenolic compounds and micro-vinification

For those accessions that produced enough grapes, were monitored their technological maturation.

From the end of August some sampling for each accession monitored were analyzed until the grape was ready to be harvest. At harvest time, sets of 10-12 bunches for thesis were sampled. Crashed bunches were used to determine the concentration of total soluble solids (°Brix) by a digital refractometer (Model 53011, TR, Forlì, Italy), the pH by a bench pH-meter (Hanna Instruments, Milano, Italy) and total acidity by a digital burette (Brand, Wertheim, Germany) by titration with NaOH 0.1 N. Also the middle weight of berry and cluster were determined.

The polyphenols richness were also tracked from the grape of the most important black accessions (Tab. 6) in the harvest of 2012.

Table 6: List of most important black accessions used to determine the polyphenols richness.

Code	Accession	Origin
2	Alberese OF 121	Alberese
23	Capalbio 1	Capalbio
24	Morcola 5 olmo	Capalbio
25	Morcola 1 edera	Capalbio
27	Morcola 3 alloro	Capalbio
30	Syl 54	Manciano
48	F. poggi	Poggi del sasso
51	Mz rossa	Poggi del sasso
53	Sangiovese	Poggi del sasso
59	Biondi melo	Sorano
60	Biondi near	Sorano
61	Del casco	Sorano
63	Piano 6	Sorano
66	Segno 1	Sorano
69	M. buono F2	Sorano

For each sampling, 60 berries were randomly chosen, divided into three groups of 20 berries, which were used as triplicates, and processed according to the Di Stefano method (Di Stefano *et al.*, 2008) slightly modified as follows.

Berry skins of each replicate were manually separated from pulp and seeds, and skins and seed were separately weighed and extracted for 4 hours at 25°C in 25 mL of a pH 3.2 tartaric buffer solution. This solution contained 12 % (v/v) ethanol, 2 g/L sodium metabisulphite, 5 g/L tartaric acid and 22 mL/L NaOH 1N. After grounding in a mortar and pestle, the extract was separated by centrifugation (R-9M: Remi Motors TD, Vasai India) for 10 min at 3000 rpm. The pellet was re-suspended in 20 mL of buffer and centrifuged for 5 minutes. The final two pooled supernatants were adjusted to 50 mL with the buffer solution. The skins extract was measured by UV-Vis absorption (Spectrophotometer HITACHI U-2000) at 540 nm after dilution (1:20) with ethanol: water: HCl (70:30:1) and at 750 nm as the seeds extract in the following solution: 0.1 mL of the extract, 6 mL H_2O, 1 mL Folin-Ciocalteu reactive, 4 mL 10% Sodium Carbonate (after 5 min) and H_2O up to 20 mL. Anthocyanins were expressed as mg of equivalents of malvidin 3-O-glucoside and phenolic compounds as mg of equivalents of (+)-catechin.

Moreover, for the accession that produced enough grape, were conducted separated micro-vinification to evaluate the value of the *V. v. sylvestris* wine, by their chemical analysis.

Therefore, we started the micro-vinification adding only few quantity [0,1 g/kg of SO_2 (sulphur dioxide)] at the begin of fermentation and also a small addition of selected yeasts (*Saccaromyces cereviseae*), following the normal technique for red-vinification.

At the end, these wines were analyzed about their: alcol content, sugar residue, total and volatile acidity, pH, dried extract, polyphenols richness and anthocyanins profile (this last was made by Dr. Giannetti from CRA-VIC Arezzo).

2.6 Molecular characterization

Traditional methodologies for the identification and the characterization of the vine's variety are of course, those ampelographyc and ampelometric, that are based on the observation of the phenotype with the detection, description, and also the measurement of morphological, phenological, physiological and productive characters of the plants. First attempt to differentiate grapevine genotypes were done by the use of iso-enzyme analysis, while in more recent times, biomolecular analysis were developed to characterize by greater objectivity in the process of data retrieval, and with a higher resolving power by the DNA analysis. Molecular characterization of 22 accessions (Tab. 7) through the analysis of the polymorphism of 9 microsatellites loci (VrZAG62; VrZAG79; VVMD25; VVMD27; VVMD28; VVMD32; VVMD5; VVMD7; VVS2) were performed at the laboratory of our Department in previous researches as reported in Italian Vitis Database (www.vitisdb.it), according to the procedure adopted by Campus (2011).

Table 7: List of the accessions on which it was made the molecular characterization.

Accession	Code	Origin
Aberese 121/2	Syl-19	Alberese
Morcola 3 alloro	Syl-16	Capalbio
Morcola 5 olmo	Syl-17	Capalbio
Capalbio 1	Syl-18	Capalbio
Ombrone 1	Syl-29	Paganico
Maschio poggi	Syl-1	Poggi del Sasso
MZ rossa	Syl-4	Poggi del Sasso
Mazzocchi 2	Syl-21	Poggi del Sasso
Alberello 96	Syl-31	Poggi del Sasso
MZ bianca	Syl-3	Poggi del Sasso
F. Poggi	Syl-2	Poggi del Sasso
Biondi 2	Syl-6-2	Sorano
Biondi 3	Syl-6-3	Sorano
Segno 1	Syl-7	Sorano
Biondi nera	Syl-74	Sorano
Piano 6	Syl-9-2	Sorano
Piano 7	Syl-10	Sorano
Cavone	Syl-11	Sorano
Del casco	Syl-12	Sorano
Montebuono F1	Syl-42	Sorano
Montebuono F2	Syl-43	Sorano
Montebuono F3	Syl-44	Sorano

2.7 Search of new accessions

During the three years period, many inspections in several areas within Tuscany Maremma, especially at the "Regional Park of Maremma" (Alberese - GR) were made. The recovery of wild vines were focused to those damp grounds, along the banks of the courses of water and in the marshy woods, that is in those areas in which the conditions were excellent for the grow of this plant. We made these searches, overall in spring and also in summer, when the vegetation of the wild vine is more apparent, and easy to recognize.

The purpose of recovery as much as possible new biotypes of *V. v. sylvestris* was very important. Every recovered plant was: marked in situ, cataloged, and mapped. Besides, when it was possible trying to interfere not too much with the natural growth of the plant, woody material or vegetative part of the plant were taken, in order to spread it and its integration into the collection, or to classified it.

2.8 Statistical analysis

The resulting data was then analyzed statistically using SPSS130 software. In detail, the ampelographyc, ampelometric, technological grape's maturation, polyphenols richness, anthocianyne profile and molecular characterization data, underwent cluster analysis and discriminating analysis. Data were subjected to multifactorial analysis with standardization, where necessary, and the visual results by centroids were visualized which report the first two canonical functions. The characteristics data of the grapes at harvest were analyzed using the MANOVA test and the differences highlighted two by two by the Tukey's test.

3 RESULTS AND DISCUSSION

3.1 Ampelographyc characterization

About the majority of the accession in the collection vineyard, it involved 55 accessions, while the other 21 were not observed because were planted in 2012 or in 2013, so they were still young (one or two years old).

The visual observations were made every year (2012, 2013 and 2014) because the different climatic conditions may be diversify the plant's organ. At the end, all data obtained were compared for each character examined, and it was extrapolated, only one or maximum two data that describes the level of expression of the accession for that character.

On table 8 are shown the ampelographyc characterization about the shoot at flowering.

Table 8: Shoot at flowering's ampelographyc characterization.

Accession	OIV CODE																	
	1	2	3	4	5	6	7	8	9	10	151	152	17	51	53	54	55	56
1	3,5	2	3	7	3	3	1	1	1	1	1	1	3	2	7	5	5,7	5
2	3	1	1	3,5	1,3	3	2	1	2	1	1	1	1,3	1,2	7	5	5,7	3,5
3	5	2	3	3	5	3	1	1	1	1	1	1	3	1,2	7	7	7	3
22	3	1,2	1	5	3	3	2	1	2	1	1	1	3,5	1,2	7	5	5,7	5
23	5	2	1	3	1	3,5	2	1	1,2	1	1	1	3	1,2	3,5	3,5	3,5	3,5
24	5	1	1	5,7	5	3	1	1	1	1	1	1	1,3	1,2	5	3,5	5	5
25	3	1	1	5	1,3	5	1	1	1	1	1	1	5	1,2	7,9	7	5,7	5
26	5	3	5	5	3	3	1	1	1	1	1	1	3,5	2,3	7,9	5,7	5	5
27	3	1	1	5,7	3	3	1	1	1	1	2	3	3,5	1,2	7,9	7	5,7	3
28	3	2	3	7	3	3	1	1	1	1	1	1	1,3	1,2	5,7	5	5	3,5
29	3	2	3,5	5	3	3	3	2	3	3	1	1	3,5	1,2	5	3	5	3
30	3	3	1	3,5	3	3	2	1	3	3	1	1,3	3	1,2	7,9	5	5	3,5
31	5	2	3	5	1	1	2	1	2	2	2	3	3,5	1,4	5,7	5	5	3,5
32	3	2	3	3	1,3	5	2	1	1	1	2	1	5	3	3,5	1,3	3	1,3
33	3	2	1,3	3,5	1	3,5	3	2	3	2	2	5	1,3	1,2	1,3	1,3	3	1
34	5	1,3	5	5	1,3	5	2	1	2	2	1	1	1	1,2	5	3	5	3

35	5	3	5	1,3	1	3,5	3	1	3	1	4	7	1,3	1,4	3,5	1	3	1
36	3	2	1,3	7	5	3	1	1	1	1	1	1	3	2	3	3	5	3
37	3	3	5	3	1	3	2	1	2	1	1	1	3	3	1	1	1	1
38	3	1	1	5	1,3	5	2	1	2	1	2	3	5,7	1,2	1,3	1	1	1,3
39	3,5	2	3	3	1,3	5	3	1,2	3	1	3	5	1,3	1,2	5,7	5	5,7	5
40	3	2	5	3	1,3	3	1	1	1	1	1	1	1	1,2	5,7	3	5	3,5
41	5	3	1	3	3	3	2	1	2	1	1	1	1	2	1	1	1	1
42	5	2	1	7	5	3	2	1	2	1	1	1	3,5	1,2	7	5	5,7	5
43	3	2	1	1	1	3	2	1	2	1	1	1	1	1	3	1	3	3
44	3,5	1	1	5,7	3,5	3	2	1	2	1	3	5	3	2	5	5	5	3,5
45	3	2	1	5	5	3	1	1	1	1	1	1	1	2	5	5	5	5
47	3	2	3	5	3	3	3	1	3	2	3	5	1	2	5	3	3	3
48	5	2	3	3	1	3	1	1	1	1	1	1	3,5	3	5	3	5	3
49	5	2	3	5,7	3	5	2	1	2	1	1	1	3,5	1,2	7	5	5,7	3,5
50	5	1,2	1	5	3	3	1	1	1	1	1	1	3,5	2	5,7	3	5,7	5
51	3	2,3	3,5	1,3	1	3	3	2	3	2	4	5	3,5	3	5	3	3,5	3
52	3,5	3	5	5	1,3	3	1	1	1	1	4	5	3,5	1,2	7	5	5,7	3
53	5	1	1	3,5	1,3	1	1	1	1	1	1	1	3	1,2	1	1	1	1
54	3,5	2	3	1	1	3	1	1	2	1	1	1	3,5	3	3	3	3	1
56	3	2	3,5	5	3	3,5	1	1	1	1	1	1	3	1	7	5	5,7	5,7
57	5	1,2	3	5,7	3	3	1	1	2	1	1	1	1,3	1,2	5,7	3	5	3,5
58	3,5	2	3	5,7	5	3	1	1	1	1	1	1	3,5	1,2	7	5	5	3
59	3	3	3,5	3,5	1	3	2	1	2	1	2	1	1,3	3	5,7	3	5	3
60	5	2	3,5	5	3	3	2	1	1	1	1	1	3,5	4	3,5	3	3,5	3
61	3	3	3	5,7	3	3	3	1	3	1	4	5,7	3,5	3	7	5	5,7	7
62	5	2	1	3,5	3	3,5	1	1	1	1	1	1	3,5	1,2	5,7	3	5	3,5
63	3,5	2	3	5	3	3	1	1	1	1	3	5	3,5	3	5	3,5	5	3
64	5	2,3	3,5	3,5	3	3	1	1	2	1	1	1	3,5	1,2	3,5	1,3	1	1
65	5	2	1	3	1	3	3	2	3	2	4	5,7	3,5	2	5,7	3	5	3
66	5	2,3	3,5	5	5	3	2	1	2	1	1	1,3	3	1,2	3,5	3	3,5	3
67	3	1,2	1	3,5	1	3	2	2	2	2	4	5	3,5	2	5	3	3,5	3
68	3	2	1,3	3	3	3	2	1	2	1	1	1	1,3	2	3,5	3	3,5	3
69	3	2	1	3	1,3	3	1	1	1	1	1	1	1,3	2,3	1,3	1	3	1

70	5	2	1	1,3	1	3	2	1	2	1	2	3	3	2	1,3	1	3	1
71	5	1	1	3	1	3	2	1	1	1	2	1	1,3	1,2	3	1	3,5	1,3
73	3	2	1	1	1	3	3	2	3	2	2	1	1	1	3	3	3	1
74	3	2	1,3	5	1,3	3	1	1	1	1	1	1	3	1,2	5	3,5	5	3
75	3	3	5	1	1	3	3	3	3	3	4	7	1,3	2	1	1	1	3
76	5	2	1	3	1	1	1	1	1	1	1	1	3	2	3	1	1	1

Discriminate analysis of the shoot characteristics has highlighted several differences which can be accounted by MANOVA (Fig. 9). In particular, the first two functions explain over 80% of total variability (Tab. 9). In this case the centroids obtained by three years of data of shoot, even though represented only by 18 parameters, shows a convergence and in the meantime several accessions are well differentiated (30, 29, 33, 51, 35 and 39).

Table 9: Eigenvalues of discriminant analysis.

Function	Eigenvalue	% of variance	Cumulated %	Canonical correlation
1	586,507	62,0	62,0	,999
2	171,478	18,1	80,2	,997
3	64,167	6,8	87,0	,992
4	37,306	3,9	90,9	,987
5	20,603	2,2	93,1	,977
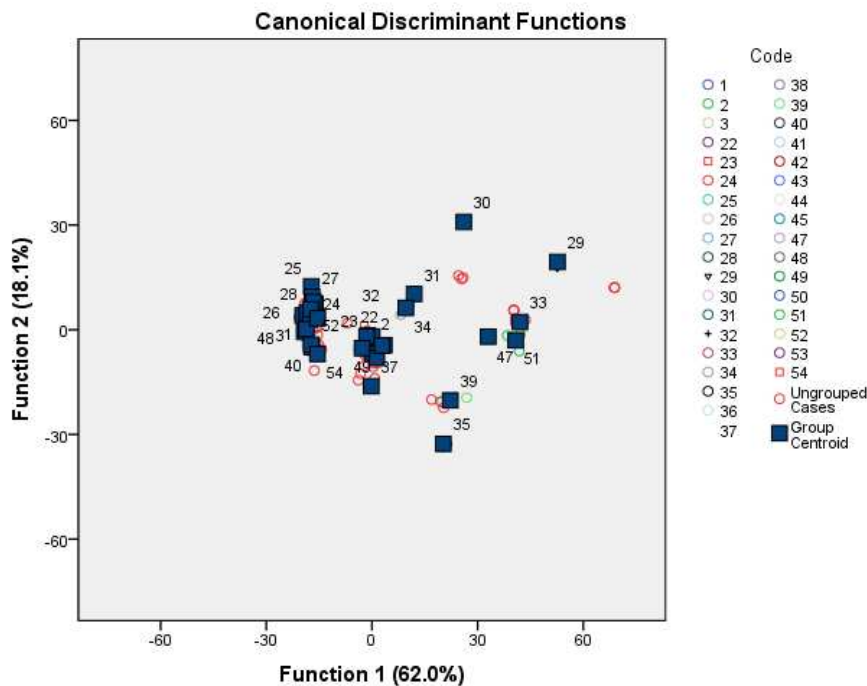
Figure 9: Centroids obtained from the cluster analysis of data recorded by OIV method on shoots at flowering along three years (2012-2014).

When cluster analysis on the average data of three years was performed, the clusters were much more dispersed and characteristics of convergence between the 75 accessions recorded showed a large diversification with similarities limited to very few accessions. Some of those coming from the same area were very close (M.buono, Piano and Segno, Biondi 1 and Biondi 3). Interesting to note how two male accessions recovered very far from them (Alberese Gr-121/2 and Maschio poggi) have very similar shoot traits (Fig. 10).

```
                  Dendrogram using Average Linkage (Between Groups)
                            Rescaled Distance Cluster Combine
            C A S E         0         5        10        15        20        25
         Label            Num  +---------+---------+---------+---------+---------+

      Mz bianco            25
      Cavone               36
      Syl 80               44
      Morcola 5 olmo        6
      Biondi 2             31
      Alberese Gr- 121/2    1
      Maschio poggi        24
      Ombrone 1 (b)        19
      Morcola 1 edera       7
      Morcola 3 alloro      9
      Biondi 1             30
      Biondi 3             32
      Morcola 2 giov        8
      Davanti cella         3
      Sforzesca syl 57     11
      Biondi melo          33
      Lionero 52           13
      Morcola 4 prunus     10
      Capannelle tardivo   21
      Syl 39               17
      Segno 1              39
      Mz 5                 27
      Piano 6              37
      Alberello 96         20
      Segno 2              40
      Cortilla lago        22
      Mz rossa             26
      Syl 53               18
      Del casco            35
      Capalbio 1            5
      Nera                 14
      Alberese O.F.-121     2
      Lionero 4 rete       12
      Montebuono F2        42
      Montebuono F3        43
      Nera 2F (sangiovese) 28
      F. poggi             23
      Biondi nera          34
      Piano 7              38
      S. filippo 2          4
      Montebuono F1        41
      Mazzocchi 2          29
      Quercia grande       15
      Rocca silvana        45
      Siepone syl 45       16
```
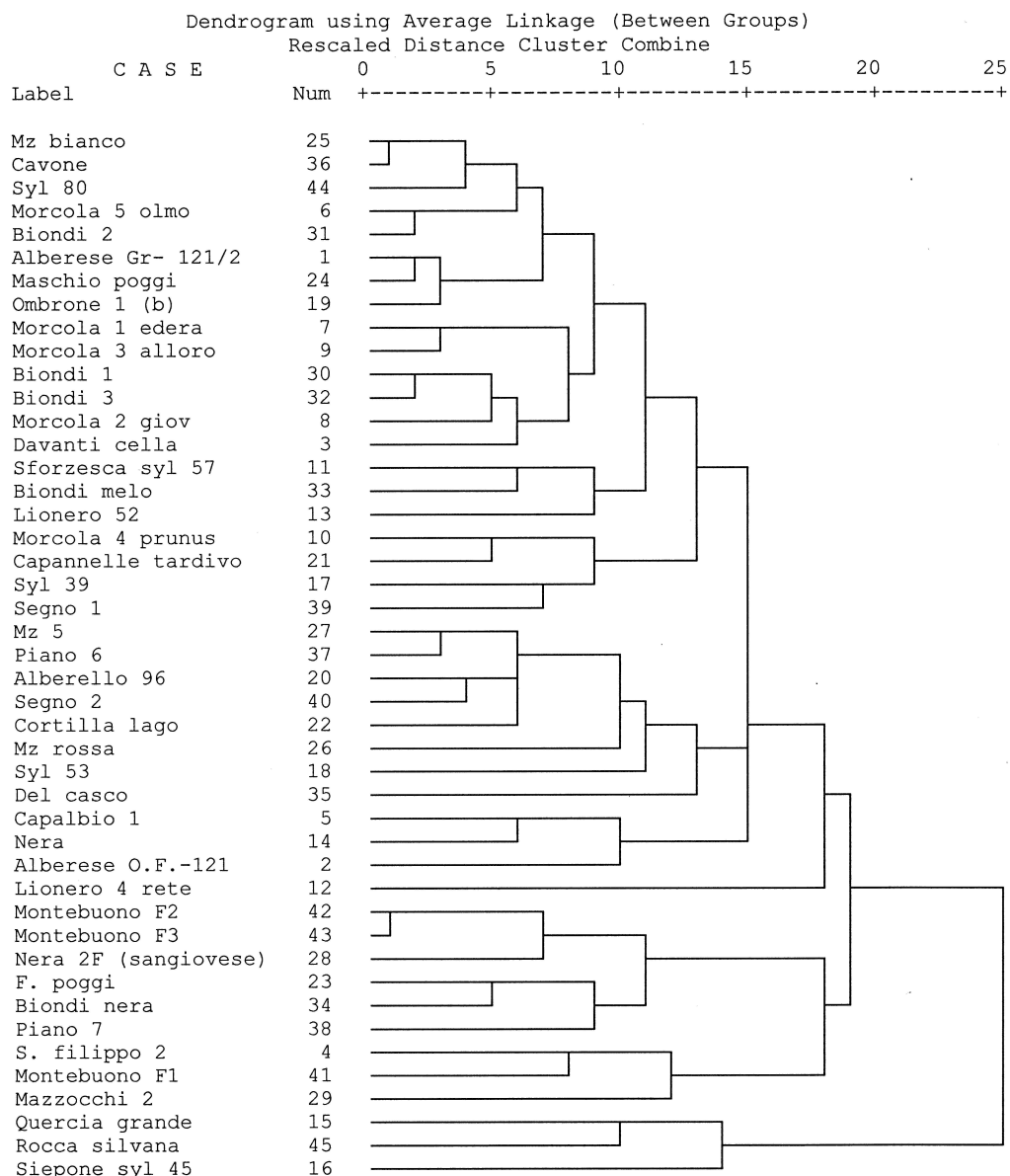
Figure 10: Cluster analysis of data recorded by OIV method on the shoots at flowering (average of three years: 2012-2014).

The most important characters that allow us to discriminate the accessions are: OIV 1 (opening of the shoot tip) and OIV 51 [color of the upper side of blade (4th leaf)].

Beyond the shoot at flowering, the ampelographyc characterization regarded the mature leaves of 75 accession. The results are listed below on table 10.

Table 10: Mature leaves' ampelographyc characterization.

Acc.	65	67	68	70	71	72	74	75	76	77	78	79	80	81-2	82	83-1	84	85	86	87	93	94
1	3,5	2	2	1	1	3	1	3,5	3	3	1,3	1,3	3	1	1	3	3	3	3	3	1,3	3,5
2	3	3	3	1	1	3	2	3	4	3	3	3	3	1	1	1	5	5	7	5	1	5
3	7,9	2	2	1	1	3	2	5	3,4	5	3	3	3	1	1	3	5	5	5	3	1	3
22	9	2	2	1	1	3	4	3	4	3	3	3	3	1	1	3	3	1	3	1	7	3
23	3	2	3	1	1	1	2	3	3	3	4	3	3	1	3	1	5	3	3	5	1,3	5
24	3,5	2	2	1	1	5,7	5	5	3	3,5	5	3	3	1	1,3	1,3	5	3	5	3,5	1	3
25	7	3	2	1	1	5	1	5	3	5,7	5	3	3	1	1	3	3	3	3	3	1	3
26	5,7	2	2	2	1,2	3	4	3	4,5	5	5	3	3	1	1	3	5	5	3,5	3	1,2	1,3
27	7,9	2	2	1,2	1	3	2	3,5	3	5	3,5	3	3	1	1	3	3,5	3	3,5	3	1,3	1,3
28	7	2	2	1	1	3,5	5	5	4	5	3,5	3	3	1	1	3	3	1	3	1	1	3
29	7	3	2	2	2	3	5	3,5	5	5	5	1,3	3	1	1	1	5	3	5	3	1	3
30	9	3	3	2	2	3	5	3,5	4	7	5,7	3	3	1	2	1	5	5	5	3	5,7	5
31	5	3	2	1	1	5	4	3	4	5	7	1	1	1	1	1	5	1	3	1	3	5
32	5,7	2	2	1	1	3	2	3	2,4	5	3,5	3	3	1	1	1	5	5	3,5	5	1	5
33	3,5	3	2	1	1	5	2	5	5	5	5	3	1	1	1	3	3	1	1	3	3	5
35	7	2	2	2,3	2	3	2	3,5	5	7	7	3	3	1	1	3	3	1	3	1	1,2	3
36	7	2	3	1	1	1,3	5	1,3	2	5	5	3	3	1	2,3	1,3	1,3	1	1	1,3	1	5
39	3	2	2	3	3	1	2	3	3	5	3	3	1	1	1	3	5	3	5	3	1	3
42	3,5	2	2	1	1	3	1	1,3	4	3	3,5	3	3	1	1	3	3	1,3	1,3	3,5	1	1,3
44	3,5	3	3	1	1	3	3	3	4	3,5	3	3	3	1	1	3	3	1	3	1	1	3
45	5,7	2	3	1	1	3	4	5,7	3	5	3	3	1	1	1	1	1	1	1	1	1	5
47	5	2	2	1	1	3	2	3	3,4	5	5	3	3	1	1	3	3	3	3	5	2	3
48	5,7	2	2	1	1	3	4	3	3	5	7	3	3	1	2,3	3	1	1	1	1	1,3	5
49	5	2	2	1	1	3	1	3,5	5	5	3,5	3	3	1	1	3	3,5	3	3	3	1	3
50	5,7	4	3	1,2	1	1,3	2	5	4	5	5,7	3	3	1	1	3	3,5	3	3,5	1,3	1	3
51	3,5	2	2	3	3	3	2	1,3	3,4	5	3	3	1	1	1	3	1	1	1	1,3	1	3
52	5	3	3	1	1	3,5	1	3,5	4	5	5	3	3	2	1	1	3	1	3	3	1	5
53	7	2	3	1	1	3,5	5	3,5	4	5	5,7	3	3	1	1	3	1	1	1	1	1	1
54	5	2	2	1	1	3	5	3	3	3	3	3	1	1	1	3	3	3	3	3	7	3
56	7	2	2	1	1	3	4	5	3	5	5	3	3	1	1	3	5	3	5	5	5,7	3
57	3,5	2	2	1	1	3,5	4	3,5	3	5	5	3	3	1	1	3	5	3	5	3,5	3	3,5
58	7	2	2	1	1	5	4	5	4	5	5	3	3	1	1	3	5	5	5	5	3	3
59	5	3	3	1	1	3	5	3	4	5	5	3	2	1	1	1	3	3	3	5	1	5
60	5	2	3	3	3	3	2	3	3	5	3	3	3	1	1	3	1,3	1	1,3	1,3	1	5
61	7	2	2	2	1	5	2	5,7	3	5	3	3	3	1	1	3	5	3	5	5	1	3
62	5	2	2	1	1	3	2	3,5	4	5	5	3	3	1	1	3	3	3	3	1	1	5
63	5	2	2	1	1	3	2	3	3,4	3	3,5	3	3	3	1	1,2	3	1,3	1,3	3	3	5
64	5,7	3	3	1	1	3	2	5	3	5	3,5	3	3	1	1	1	1	1	1,3	1,3	5	5

66	9	3	3	1	1	3,5	4	3,5	4	5	3,5	3	3	1	1	1	5	1,3	1,3	3,5	1	5
67	5	3	3	1	1	5	1	5	4	3	3	3	3	1	1	3	3	3	3	3	1	3
68	9	2	2	1	1	5	1	3,5	3	3	3	3	1	1	1	3	1,3	1	1	1,3	7,9	3
69	3,5	2	2	1	1	3,5	4	3,5	3,4	3	3	3	3	1	1	3	1	1	1	3	1	3
70	3,5	2	2	1	1	1	4	3,5	4	3	3	3	3	1	1	3	1	1	1	3	1	3
74	5	2	2	2	2	3	4	3,5	3	5	3	3	3	1	1	3	5	3	3	3	2	3
75	3	2	2	1	2	3,5	1	5	3	5	3,5	3	3	1	1	1	1	3	3	5	2	5

Discriminate analysis of the leaf characteristics analyzed (twenty-five codes) by MANOVA (Fig. 11) evidenced a large variability between accession and also between the three years, as was expected, because of the environmental differences. In this case, the centroids obtained reported for the first two functions about 70% of the total variability explained, leaving ungrouped several cases (Tab. 11).

Table 11: Eigenvalues of discriminant analysis.

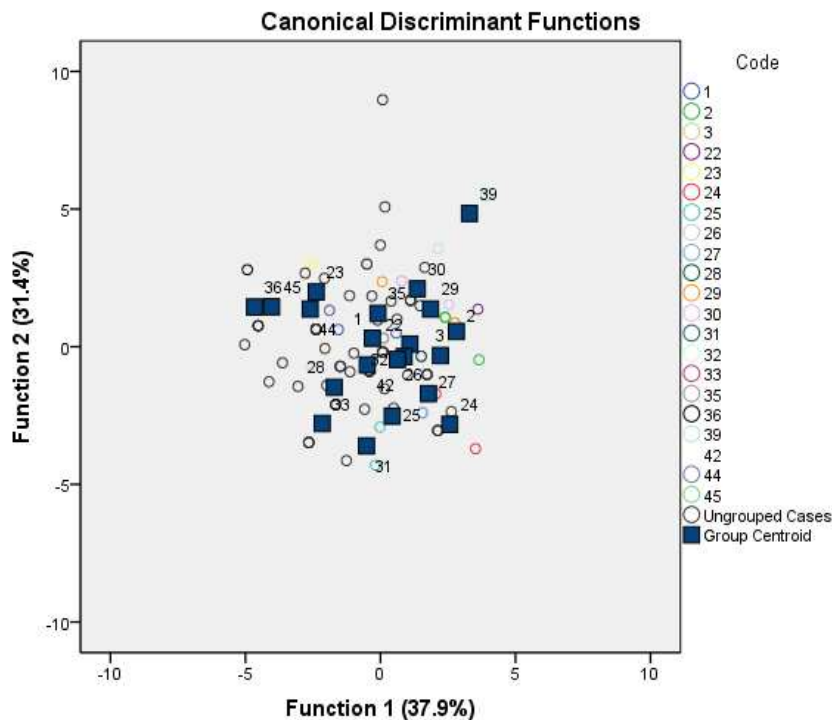| Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	7,137	37,9	37,9	,937
2	5,900	31,4	69,3	,925
3	3,939	20,9	90,3	,893
4	1,386	7,4	97,6	,762
5	,446	2,4	100,0	,555

Figure 11: Centroids obtained from the cluster analysis of data recorded by OIV method on fully expanded leaves along three years (2012-2014).

When cluster analysis was performed on average data recorded along three years over mature leaves, the 75 accessions were spreadly grouped and in few exceptions were placed like for the shoot cluster (Montebuono F1 and F2). Most of the accessions were ranked irrespectively to the group regarding the shoot and the area of origin, except Cortilla lago and M. poggi, Biondi 1 and Biondi 3 (Fig. 12).
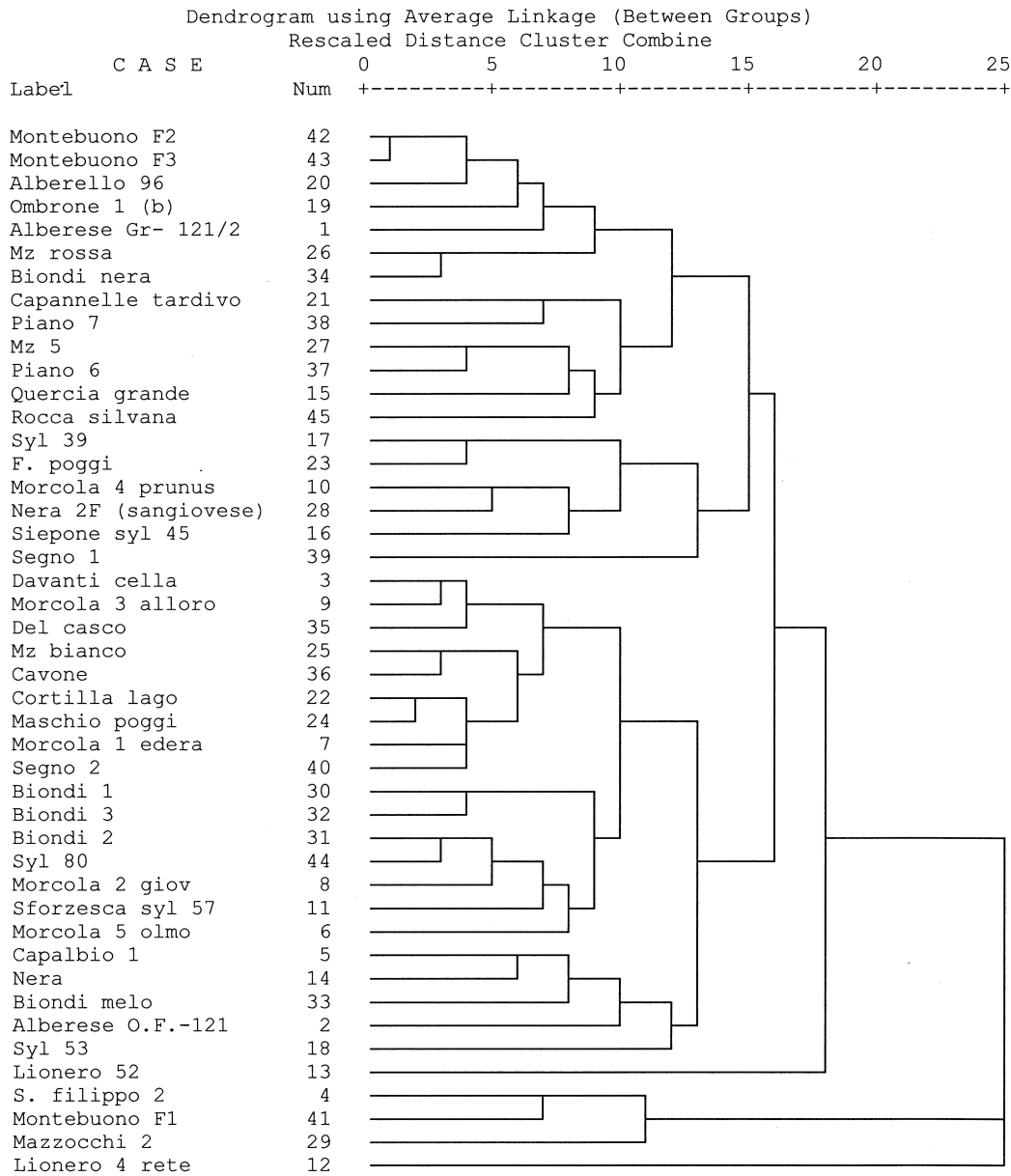
```
              Dendrogram using Average Linkage (Between Groups)
                      Rescaled Distance Cluster Combine
            C A S E              0         5        10        15        20        25
          Label               Num  +---------+---------+---------+---------+---------+

          Montebuono F2        42
          Montebuono F3        43
          Alberello 96         20
          Ombrone 1 (b)        19
          Alberese Gr- 121/2    1
          Mz rossa             26
          Biondi nera          34
          Capannelle tardivo   21
          Piano 7              38
          Mz 5                 27
          Piano 6              37
          Quercia grande       15
          Rocca silvana        45
          Syl 39               17
          F. poggi             23
          Morcola 4 prunus     10
          Nera 2F (sangiovese) 28
          Siepone syl 45       16
          Segno 1              39
          Davanti cella         3
          Morcola 3 alloro      9
          Del casco            35
          Mz bianco            25
          Cavone               36
          Cortilla lago        22
          Maschio poggi        24
          Morcola 1 edera       7
          Segno 2              40
          Biondi 1             30
          Biondi 3             32
          Biondi 2             31
          Syl 80               44
          Morcola 2 giov        8
          Sforzesca syl 57     11
          Morcola 5 olmo        6
          Capalbio 1            5
          Nera                 14
          Biondi melo          33
          Alberese O.F.-121     2
          Syl 53               18
          Lionero 52           13
          S. filippo 2          4
          Montebuono F1        41
          Mazzocchi 2          29
          Lionero 4 rete       12
```

Figure 12: Cluster analysis of the data recorded by OIV method on mature leaves (average of three years: 2012-2014).

Lastly, the ampelographyc characterization of cluster, berry and seed, which involved 20 accessions (those female that produced significantly) of the collection vineyard, reported on table 12 (cluster's characterization); table 13 (berry and seed's characterization) and table 14 (some agronomic dates), confirmed the large variability of the cluster characteristics except for two accessions found in different places (S. filippo 2 and Segno 1) and relatively close (Piano 6 and M. buono F1).

Table 12: Cluster's ampelographyc characterization.

Accession	OIV CODE						
	202	203	204	206	207	208	209
2	3	1	3	3	5	2	2
22	3	1	9	3	1	2	1
23	3	3	5	3	1	1	2
24	3	1	3	5	5	1	1
25	3	1	5	3	1	2	1
26	3	3	5	3	1	2	2
27	3	1	5	1	1	2	2
30	3	1	7	1	7	1	1
41	3	1	3	1	1	3	1
48	5	3	3	3	1	2	2
50	5	1	5	3	1	2	1
51	5	1	3	5	5	1	1
53	7	3	5	5	1	2	2
56	3	3	5	3	7	3	2
59	3	1	9	3	5	2	1
60	3	1	7	3	1	2	1
61	3	3	7	3	5	3	2
63	1	1	7	1	5	1	1
66	7	3	5	5	5	1	1
68	3	1	7	3	5	1	1

Table 13: Berry and seed's ampelographyc characterization.

Acc	OIV CODE																			
	220	221	222	223	225	226	227	228	229	231	232	233	235	236	238	240	241	242	243	244
2	1	1	2	2	6	2	3	1	1	3	1	3	1	1	3	2	2	1	1	1
22	1	1	2	2	6	2	5	5	1	3	2	3	2	1	1	3	3	3	1	1
23	3	3	2	1	6	2	3	5	1	1	1	3	1	1	1	3	3	1	1	1
24	1	1	2	2	6	2	7	3	1	1	1	3	1	1	3	3	3	3	1	1
25	1	1	1	2	6	2	7	5	1	5	2	3	1	1	3	2	3	3	1	1
26	1	1	1	7	1	2	5	5	1	1	1	3	1	1	3	2	3	3	1	1
27	1	1	1	2	6	2	5	5	2	3	1	3	1	1	1	2	3	1	1	1
30	1	1	2	2	6	2	5	3	1	3	1	3	1	1	1	3	3	1	1	1
41	1	1	2	2	1	2	5	5	2	1	1	3	1	1	3	2	3	3	1	1
48	3	3	2	2	6	2	5	5	1	5	2	3	2	1	5	2	3	1	1	9
50	3	3	1	2	1	2	3	1	1	1	2	3	1	1	3	2	3	3	1	1
51	1	1	2	2	6	2	5	5	1	3	1	3	2	1	1	2	3	3	1	1
53	3	3	1	2	6	2	5	5	1	3	2	3	1	1	5	2	3	1	1	1
56	1	1	2	2	1	2	3	5	2	1	2	3	2	1	1	2	3	3	1	1
59	1	1	2	2	6	2	5	5	1	5	2	3	1	1	1	3	3	5	1	1
60	1	1	1	2	6	2	3	3	1	3	2	3	1	1	1	1	3	3	1	1
61	1	1	1	2	6	2	5	5	1	1	2	3	1	1	1	3	3	3	3	1
63	1	1	2	2	6	2	5	3	1	3	1	3	2	1	1	3	3	3	1	1
66	1	1	2	2	6	2	5	7	1	3	1	3	3	1	3	3	3	1	1	1
68	1	1	2	2	6	2	5	3	1	3	1	3	1	1	1	2	3	3	1	1

Table 14: Agronomic parameters.

Accession	OIV CODE			
	304	502	503	504
2	7	1	1	1
22	5	1	1	1
23	5	1	1	1
24	5	1	1	3
25	5	1	1	3
26	5	1	1	3
27	5	1	1	5
30	5	1	1	1
41	5	1	1	3
48	5	1	1	5
50	5	1	1	3
51	5	1	1	3
53	5	1	3	1
56	5	1	1	1
59	5	1	3	1
60	5	1	1	1
61	5	1	1	5
63	5	1	1	1
66	5	1	1	1
68	5	1	1	1

Cluster analysis showed as the female accession were diversify in the different group (Fig. 13).

```
              Dendrogram using Average Linkage (Between Groups)
                    Rescaled Distance Cluster Combine
           C A S E            0         5        10        15        20        25
        Label            Num   +---------+---------+---------+---------+---------+

        S. filippo 2       2
        Segno 1           19
        Morcola 3 alloro   7
        Mz rossa          12
        Morcola 1 edera    5
        Biondi nera       16
        Capalbio 1         3
        Piano 6           18
        Montebuono F1     20
        Lionero 4 rete     8
        Biondi melo       15
        Del casco         17
        Alberese O.F.-121  1
        Morcola 5 olmo     4
        Nera 2F (sangiovese) 13
        Morcola 2 giov     6
        3 Cantoni          9
        Mz bianco         11
        Biondi 1          14
        F. poggi          10
```

Figure 13: Cluster analysis of data recorded on cluster, berry and seed at harvest by OIV method.

The most important characters that allow us to discriminate the accessions for this characterization are: OIV 202, 203, 204, 208, 209 (bunch's length, width, density, shape and number of wings), OIV 220, 221, 223 (berry: length, width and shape).

At this point, given the accessions' distribution in different groups also for accessions recovered in the same area, we can hypothesized that the natural cross has contributed to generate large variability in the progeny.

3.2 Ampelometric characterization

From the statistical analysis of the data obtained with the leaf characterization through the software "SuperAmpelo" (Fig. 14), so an objective characterization that was not affected by the operator's subjectivity, we found three distinct groups. The first one contains 12 accessions from Biondi melo to Lionero 54 coming from different area (five of them were recovered from Sorano). The second includes 9 accessions from Morcola 3 to Morcola 2, coming from Capalbio, Cinigiano and Sorano. The last group (12 accessions) from Capalbio 1 to Alberese gr 121 contains accession coming from all the area of recovered, and particularly all those found in Alberese area.

```
            Dendrogram using Average Linkage (Between Groups)
                    Rescaled Distance Cluster Combine
        C A S E           0         5        10        15        20        25
   Label               Num  +---------+---------+---------+---------+---------+

   biondi melo           7   ─┐
   il cavone            12   ─┤
   biondi 3              6   ─┤
   nera 2f              29   ─┼─┐
   mz bianco            26   ─┤ │
   syl 39               32   ─┤ │
   femmina poggi        11   ─┤ ├───────┐
   ombrone 1            30   ─┘ │       │
   il piano 6           13   ─┐ │       │
   morcola 1            21   ─┤ │       │
   il piano 7           14   ─┼─┘       ├───────────────────────────────┐
   lionero syl 54       34   ─┤         │                               │
   siepone syl 45       33   ─┘         │                               │
   morcola 3alloro      23   ─┐                                         │
   nera                 28   ─┤                                         │
   del casco            10   ─┤                                         │
   il segno 1           15   ─┼─┐                                       │
   maschio poggi        20   ─┤ │                                       │
   Mazzocchi 2          31   ─┤ │                                       │
   morcola 4 prunus     24   ─┤ ├─────────────────┐                     │
   m buono f1           17   ─┤ │                 │                     │
   morcola 2giov        22   ─┘ │                 │                     │
   capalbio 1            9   ─┐ │                 ├─────────────────────┘
   m buono f2           18   ─┼─┤                 │
   biondi 2              5   ─┘ │                 │
   alberello 96          1   ─┐ │                 │
   alberese of 121       3   ─┤ │                 │
   biondi 1              4   ─┼─┤                 │
   m buono f3           19   ─┤ │                 │
   biondi nera           8   ─┤ │                 │
   morcola 5 olmo       25   ─┘ │                 │
   il segno 2           16   ─┐ │                 │
   mz rosso             27   ─┼─┘                 │
   alberese gr 121       2   ─┘                   │
```

Figure 14: Cluster analysis of data obtained by leaves measured by "SuperAmpelo" software.

3.3 Pathological monitoring

The phytosanitary study showed that fungal diseases, such as downy mildew (*Plasmopara viticola*) cause symptoms on all the observed populations. The accession were grouped according to the level of leaves' infection suffered. The major of the accessions appeared quite susceptible to this infection, because more than 68% of these suffered an infection that compromised from 25 to 75% of the leaves' surface (Tab. 15). In fact, only 1/3 (31%) of the examined accession showed to have suffered a level of infection less than 25% of the leaf surface. Anyhow, the infection suffered by *V. v. sylvestris* was less or the same of that suffered by cv. Sangiovese (accession n° 53) as control, that suffered the highest infection's level (Bouby *et al.,* 2013; Ocete *et al.,* 2011).

Table 15: Results of downy mildew's attack.

Infection's level	14 Accession	Infection's level	18 Accession	Infection's level	13 Accession
4 (<25%)	1	5 (<50%)	2	6 (<75%)	3
	23		26		22
	24		28		44
	25		29		47
	27		33		49
	30		36		51
	31		39		53
	32		42		54
	35		45		56
	63		48		59
	67		50		61
	70		52		68
	74		57		69
	75		58		
			60		
			62		
			64		
			66		

In the second pathological test, the screening revealed the following situation: on 44 accessions analyzed only 6 (13.3 %) showed the presence of infective states singly or in combination of 4 of the 9 viral agents searched (ArMV, GFLV, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV 7, GVA, GVB and GFkV). In particular, serological assays revealed the presence of infections due to GLRaV-1, GLRaV-3, GVA and GVB, and in no case were highlighted strains positive of ArMV, GFLV, GLRaV 2, GLRaV 7 and GFkV.

From the 6 positive accessions, 4 were infected by GLRaV 1, and 2 of these showed, moreover, contemporary infectious states supported by GVA. The other 2 accessions were positive infected from single infections determined respectively, from GLRaV 3 and GVB (Tab. 16).

Table 16: Enzyme immunoassays investigations' results.

Accession	Origin	Virus agent discovered with the ELISA test			
		GLRaV 1	GLRaV 3	GVA	GVB
1	Alberese	+	-	+	-
2	Alberese	+	-	+	-
49	Poggi del sasso	-	-	-	+
56	Sorano	-	+	-	-
62	Sorano	+	-	-	-
64	Sorano	+	-	-	-
Total		**4**	**1**	**2**	**1**

Before now, never in literature was verified the presence of these two *vitivirus* (GVA and GVB) and mixed infections on *V. v. sylvestris.*

The viral infection of the two *ampelovirus* (GLRaV 1 and GLRaV 3) may have been transmitted through the trophic actions of vector species (coccidi and/or pseudococcidi), because the accession infected were found near vineyard of *V. v.* subsp. *sativa*. As mentioned by Di Vecchi *et al.,* (2009) in many cases, wild grapevine have been identified near vineyards.

3.4 Thermal requirements for bud-breaking

During the 2013 winter period, under the environmental conditions of the Tuscany Maremma, temperatures led to a satisfactory amount of Chill Units (CU).

Beginning from the 20/11/2012 (the end of the leaves fall) to the 1st JD, plants have already accumulated 627 CU. After which, 800 CU were recorded on 10 JD, 1000 CU on 20 JD and 1200 CU on 30 JD (Fig. 15).
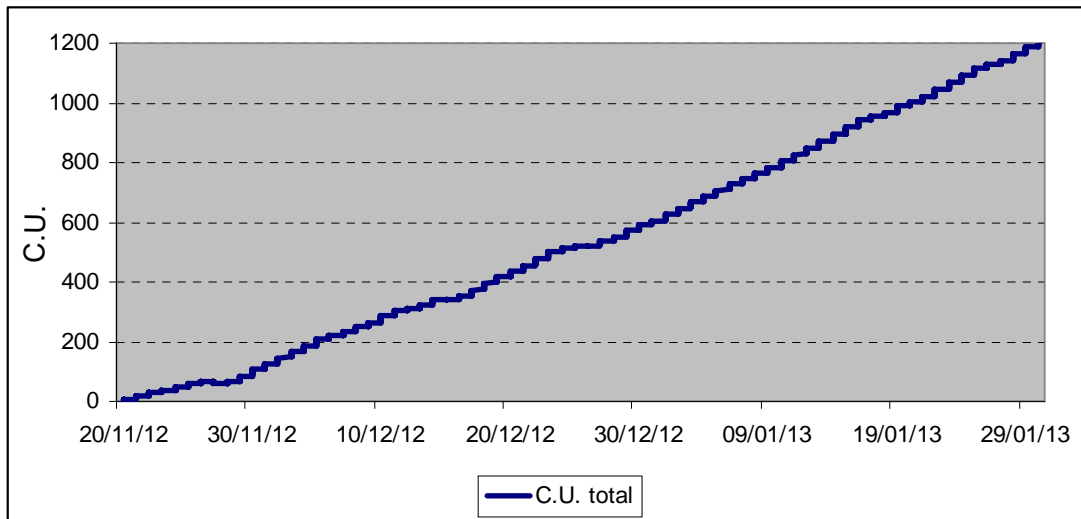
Figure 15: Chill Units accumulated from 20/11/2102 to 31/01/2013 in field.

Departing from the hourly temperatures (Fig. 16) recorded by automatic data-loggers, when the major of the buds reached the stage 03, both in field and in forcing condition, were calculated the GDH amount, through the Richardson's method, which were demanded, and these values were compared to show any differences between the accessions in field and in forcing conditions.
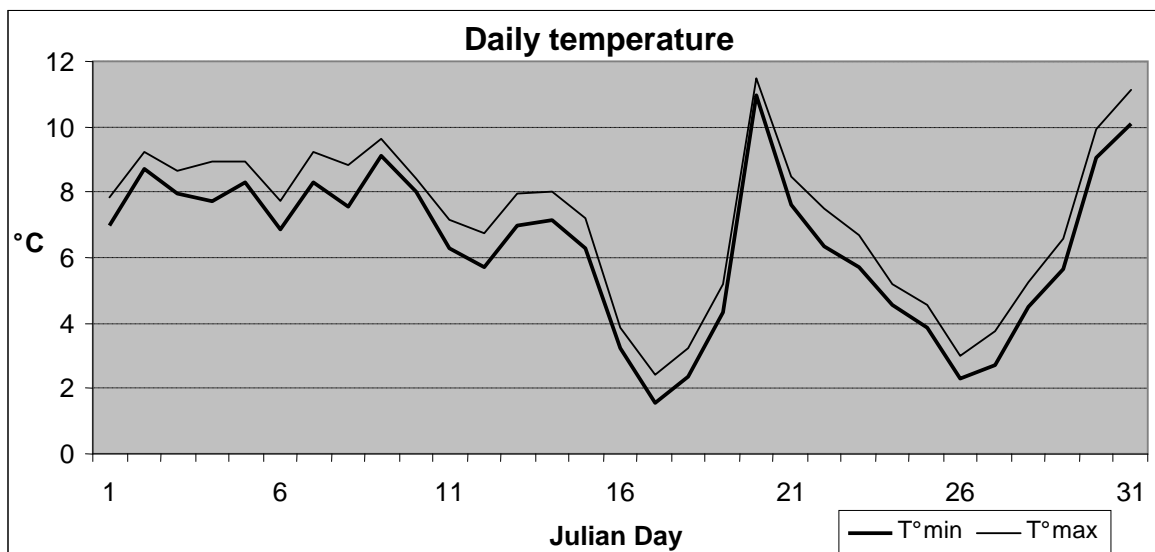
Figure 16: Daily (minimum and maximum) temperatures recorded during winter 2012 at ColleMassari farm.

In field conditions, according to the different GDH accumulation (Fig. 17), it was possible to characterize in three different groups (earlier, early, intermediate) the accession tested (Fig. 18).
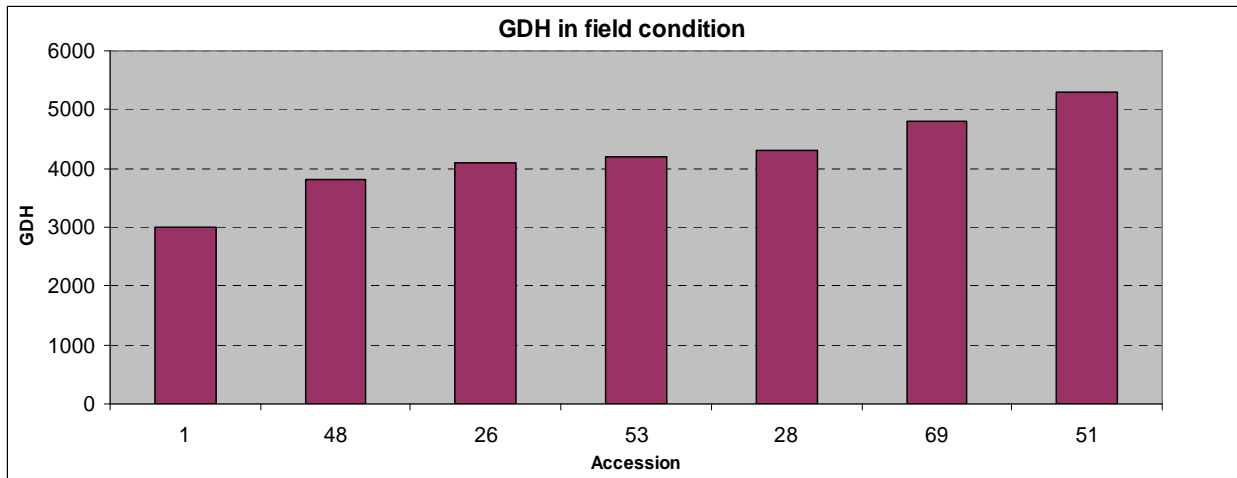
Figure 17: GDH amount for each accession in vineyard.
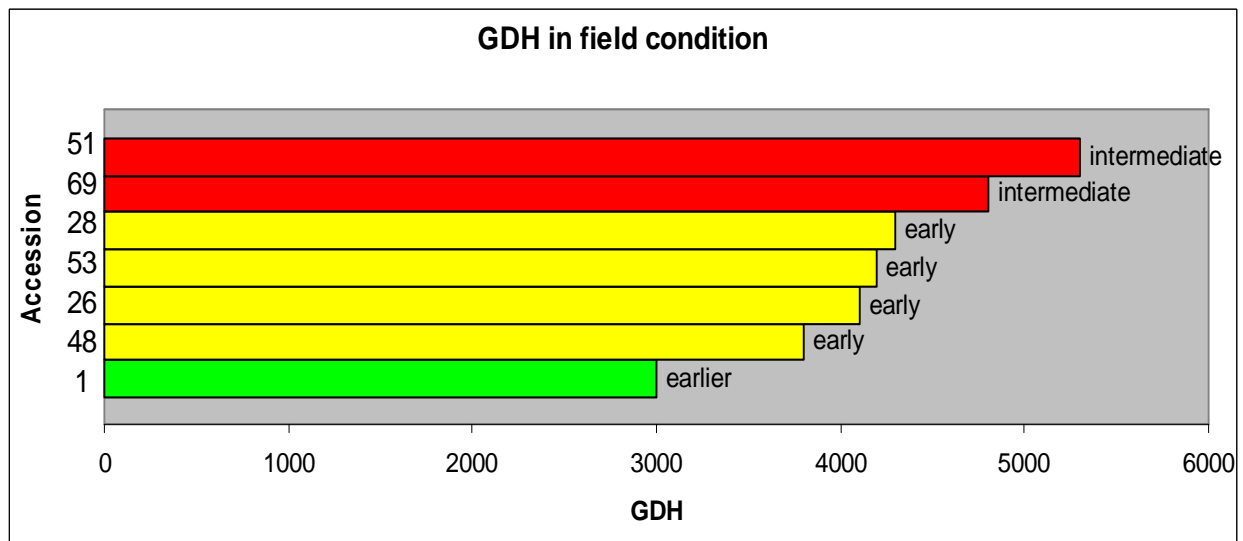
Figure 18: Classification of the accession about the GDH received in field to reach bud break.

This classification was possible taking into consideration as reference the cv. Sangiovese (accession n° 53) that is considered an early variety.

Under forcing conditions, the bud development showed a different behaviour in comparison with natural conditions (Fig. 19).
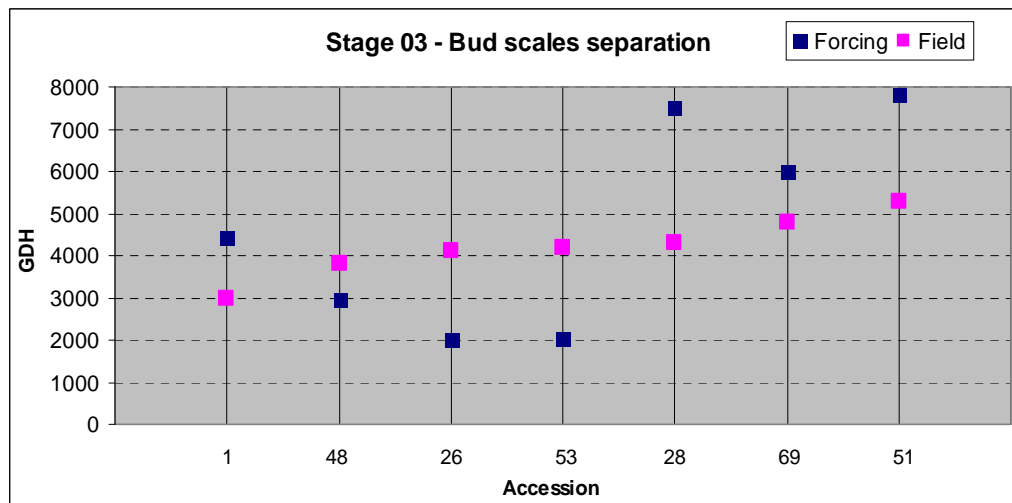
Figure 19: Comparison of the GDH demand of the accession between field and forcing conditions.

In fact, the constant temperature of the forcing condition could have a different action according to the stage that the bud reached in field until the drawing. Moreover, lengthy stays in the cold, as occurred in the winter of 2013, reduces the need of warm of the bud itself. However, some accessions need of more hours of warm in filed, other in forcing conditions. When compared with geographic distribution of species and genotypes, patterns suggest that chilling requirement and budburst rate are adaptive traits (Londo and Johnson, 2014).

During the winter period, every time (four samples: 1) 14 Dec.; 2) 21 Gen.; 3) 8 Feb.; 4) 1 Mar.) that were taken the buds was determined the middle weight of the same before and after forcing, to evaluate the time when a substantial weight's change occurred (Fig. 20).

Differential response to field and forcing conditions were noted between the accessions studied. Accessions 69, 26, 28 and 31 maintained unchanged the bud weight in natural environment along the first three dates of sampling (from 14 Dec. to 8 Feb.), followed by an increase of bud weight in field and in forcing conditions at the 4th sample date (1 Mar.), showing thus to have overcome the endo-dormancy. Accessions 1, 48 and 53 showed a gradual increase in field conditions as the time proceeded, and warm conditions of forcing stimulated the growth capability, exhibiting a different mode to respond to temperatures if compared to the other genotypes. Given this different behaviour we could expect to have also a variability on phenology especially the phenophase of bud breaking as reported on figure 21.
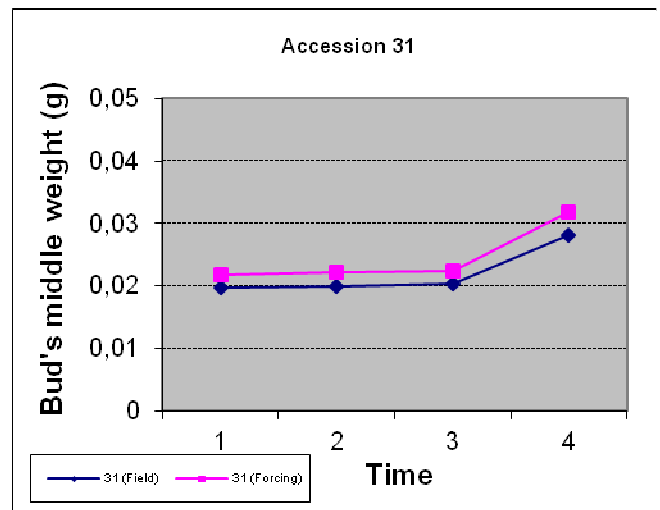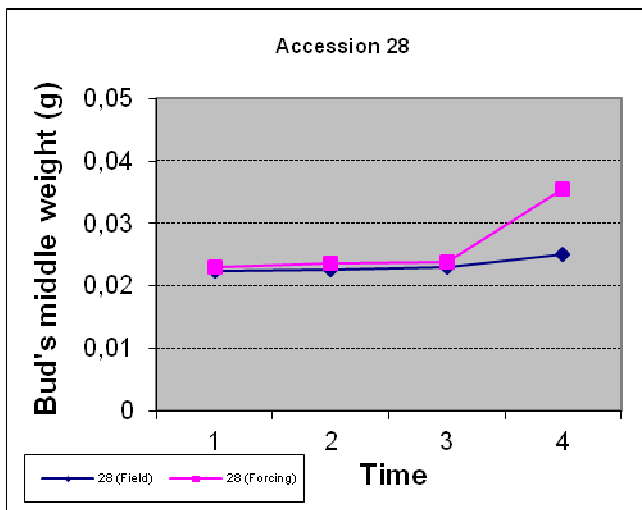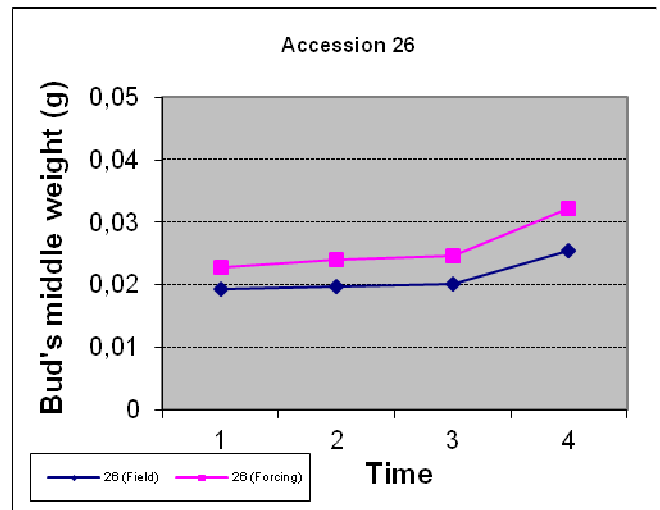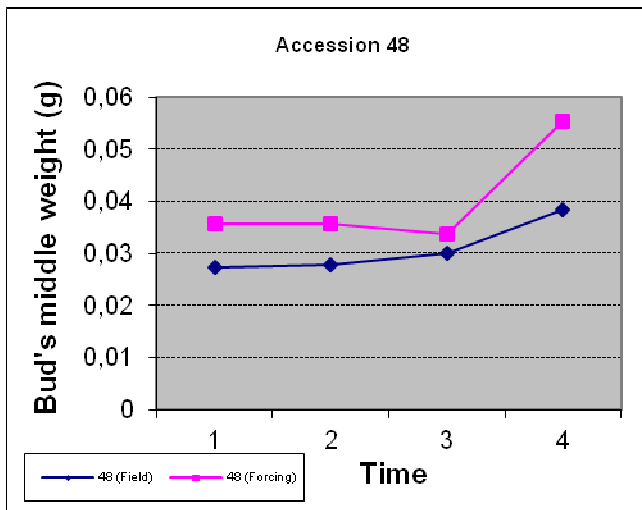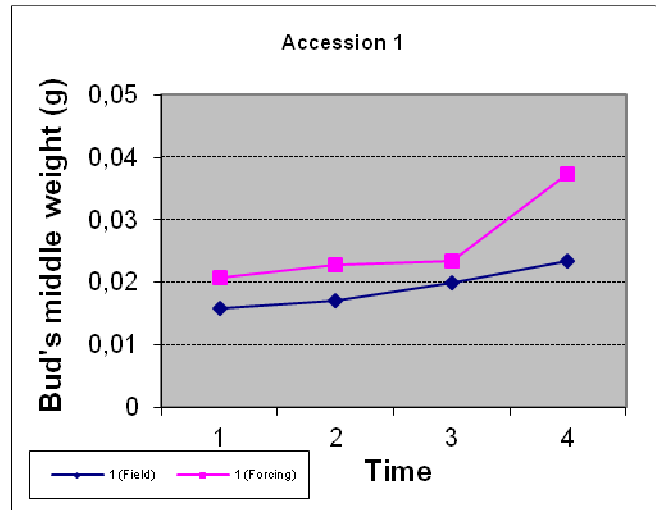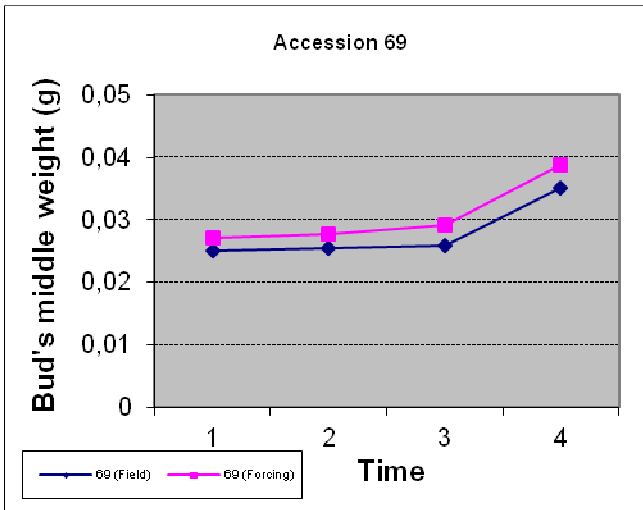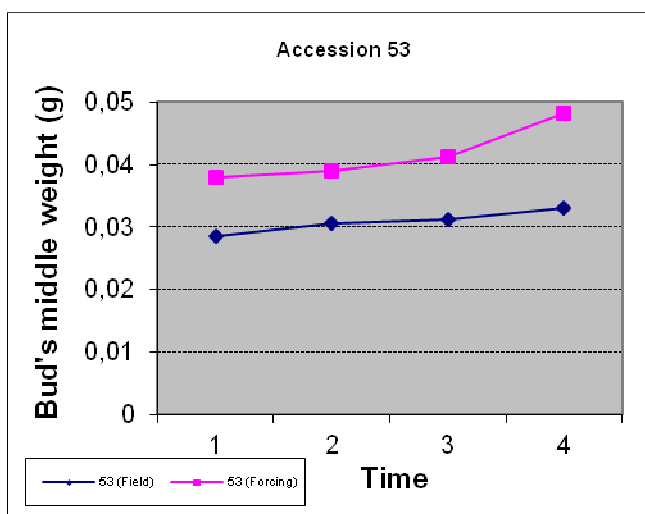
Figure 20: Middle weight of the seven accession utilized in the forcing.

As shown by graphs all accessions expressed significant weight's increasing both in field and forcing at time 3. From comparison of the mean stage of budding between 2012 and 2013 (Fig. 21) is evident how in different climatic years, the climate's action changes the behaviour of the accessions that at the same observation time (100th Julian day) appear reached stages always different.
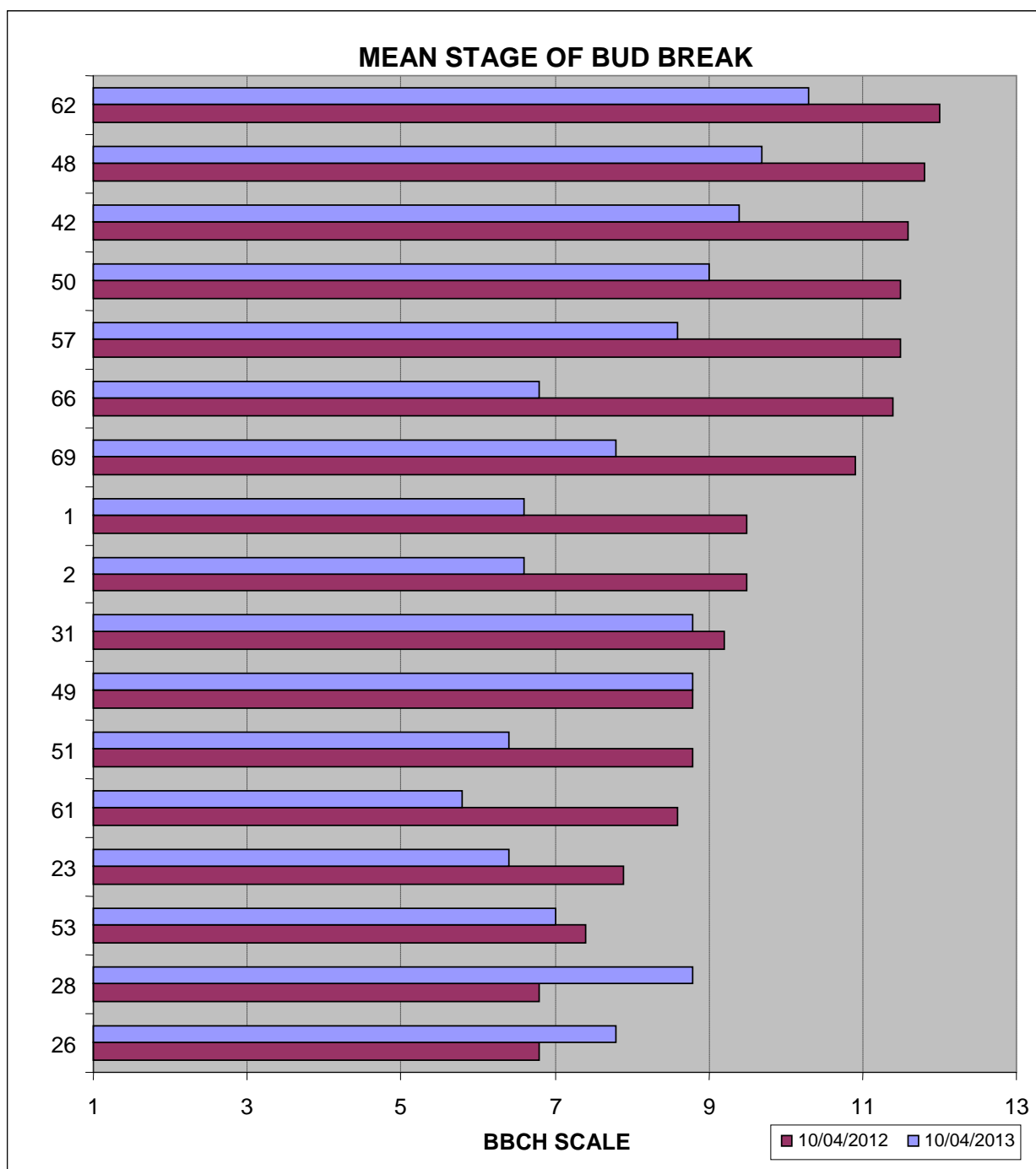
Figure 21: Comparison of the mean stage of bud-break between 2012 and 2013 seasons.

The major of these accessions during 2012 reached more advanced level about the BBCH scale respect to 2013. But, the difference are very variable. The accession 49 show no differences within the year examined, while accessions 28 and 26 reached level more advanced level in 2013.

3.5 Grape analysis, phenolic compounds and micro-vinification

Some interesting accession (15 with black berry and 3 with white berry), that produced enough grapes of sufficient quality were monitored at maturity (Tab. 17).

Table 17: Technological maturation of the grape of some accessions at harvest.

Accession	Color	Origin	M. wgt grape (g)	M. wgt berry (g)	°Brix	Acidity (g/L)	pH
ALBERESE O.F.-121	Black	Alberese	14	0.476	26.5	7.72	3.08
CAPALBIO 1	Black	Capalbio	11	0.456	25.4	9.27	3.22
MORCOLA 5 OLMO	Black	Capalbio	19	0.652	24.2	7.27	2.96
MORCOLA 1 EDERA	Black	Capalbio	29	0.553	23.4	10.76	3.02
MORCOLA 3 ALLORO	Black	Capalbio	29	0.463	24.8	8.81	3.00
SYL 54	Black	Manciano	24	0.465	19.3	12.14	2.87
F. POGGI	Black	Poggi del sasso	36	0.674	23.2	8.67	3.11
MZ ROSSA	Black	Poggi del sasso	20	0.684	20.5	11.8	2.77
NERA 2F	Black	Poggi del sasso	54	0.813	22.4	6.96	3.11
BIONDI MELO	Black	Sorano	18	0.521	22.5	12.34	2.81
BIONDI NERA	Black	Sorano	9	0.327	25.1	12.29	3.14
DEL CASCO	Black	Sorano	34	0.513	19.8	9.01	2.94
PIANO 6	Black	Sorano	25	0.498	18.9	11.24	3.01
SEGNO 1	Black	Sorano	24	0.637	22.0	9.41	2.90
M.BUONO F2	Black	Sorano	12	0.515	23.5	7.55	3.29
MORCOLA 2 GIOV	White	Capalbio	35	0.675	23.4	11.96	2.87
OMBRONE 1	White	Paganico	14	0.585	22.6	5.26	3.47
MZ BIANCA	White	Poggi del sasso	27	0.936	19.9	6.35	3.24

These female accessions reached good level of technological maturation, even if very variable. As peculiarity, were very low the value about the middle weight of both grape (always lower of 60 g) and berry (lower 1 g), as confirmation with the literature of this subspecies. In fact, these values are smaller than in cultivated grapes, which usually weigh more than 1000 mg (Revilla *et al.,* 2012). Satisfactory results the sugar concentration, while in some cases pH values are quite low and the acidity high.

These dates were statistically analyzed and the dendrogram (Fig. 22) show the similarity between the accessions.
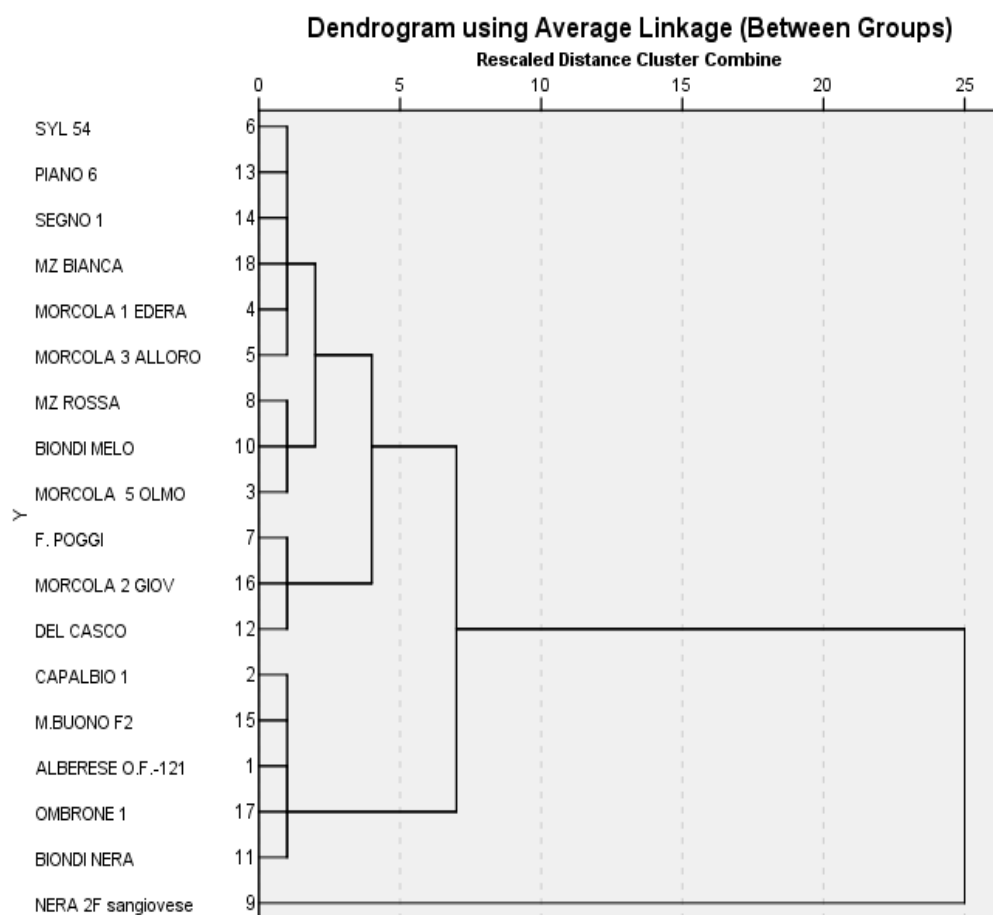
Figure 22: Dendrogram obtained with the statistical analysis of the technological maturation of the grape.

Besides the various clusters formed by the accessions of *V. v. sylvestris* analyzed, it is more important underline the hierarchical distance that exists between all these and the cv. Sangiovese. Moreover, from the accessions whit black berry, were determined the phenolic richness. For this analysis the anthocyanins were extracted from the skin and polyphenols from skin and seed of every accession.

The results of this analysis (Fig. 23) show the particular richness of anthocyanins, expressed as malvidin in the skin, of all the accession of *V. v. sylvestris* respect to the reference (*V. v. vinifera* cv. Sangiovese), coming from the same area and inserted at the end of the graphic. In the wild accessions under study, the content of total anthocyanins was too high when it was expressed in mg·kg^{-1} of grapes, with values exceeding sometimes 2000 mg·kg^{-1} grapes. When comparing these values with data obtained by the authors in the same Germplasm Bank, the total content of anthocyanins in wild grapes, in mg·kg^{-1} grapes basis, was higher than in many cultivated grapes. This effect is due to the average size of the grapes, too small in wild accessions. In some case the concentration of these components were very high (accession: 24, 48, 51, 59, 61, 63, 66).

The results obtained indicate that the content of the skins and seeds' polyphenols of these accessions have a large variability.
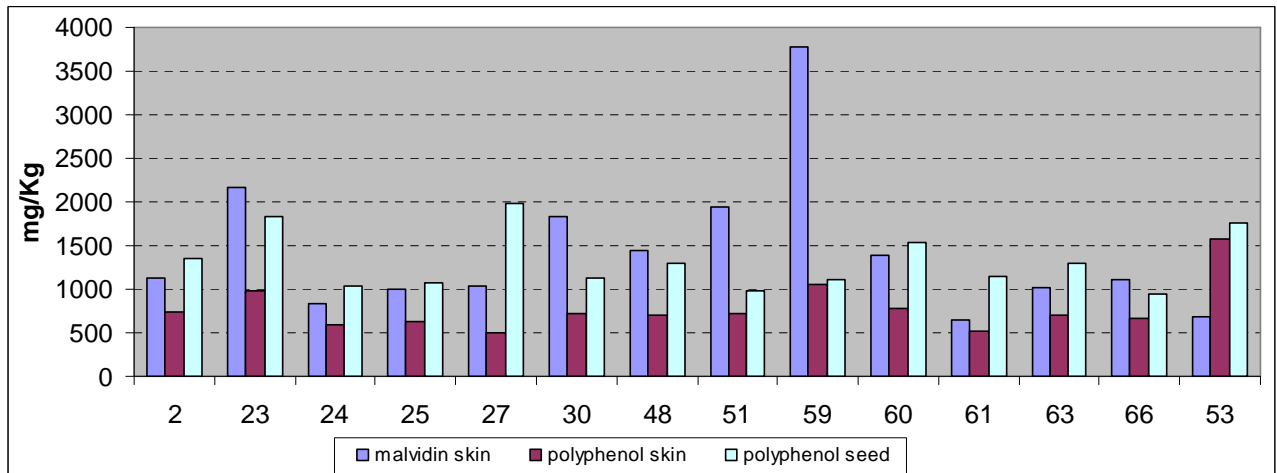
Figure 23: Polyphenol richness of several accessions.

These dates were also statistically analyzed to compare the accessions (Fig. 24). As results, a part the more or less marked difference between the accessions, that were grouped according to their similarity as previously mentioned, all these show important differences from the profile of the cv. Sangiovese.

The comparison of the anthocyanins profile between the cv. Sangiovese and the *V. v. sylvestris* more representative show the heavy differences about their percent composition (Fig. 25 – 26). In the first, the profile is much more equilibrate with prevalence of malvidin follow by cyanidin and peonidin. About the second, is always the malvidin the most prevailing, but in this case it is very predominant (68.5 %). Moreover in this last, cyanidin and peonidin are very low represented.
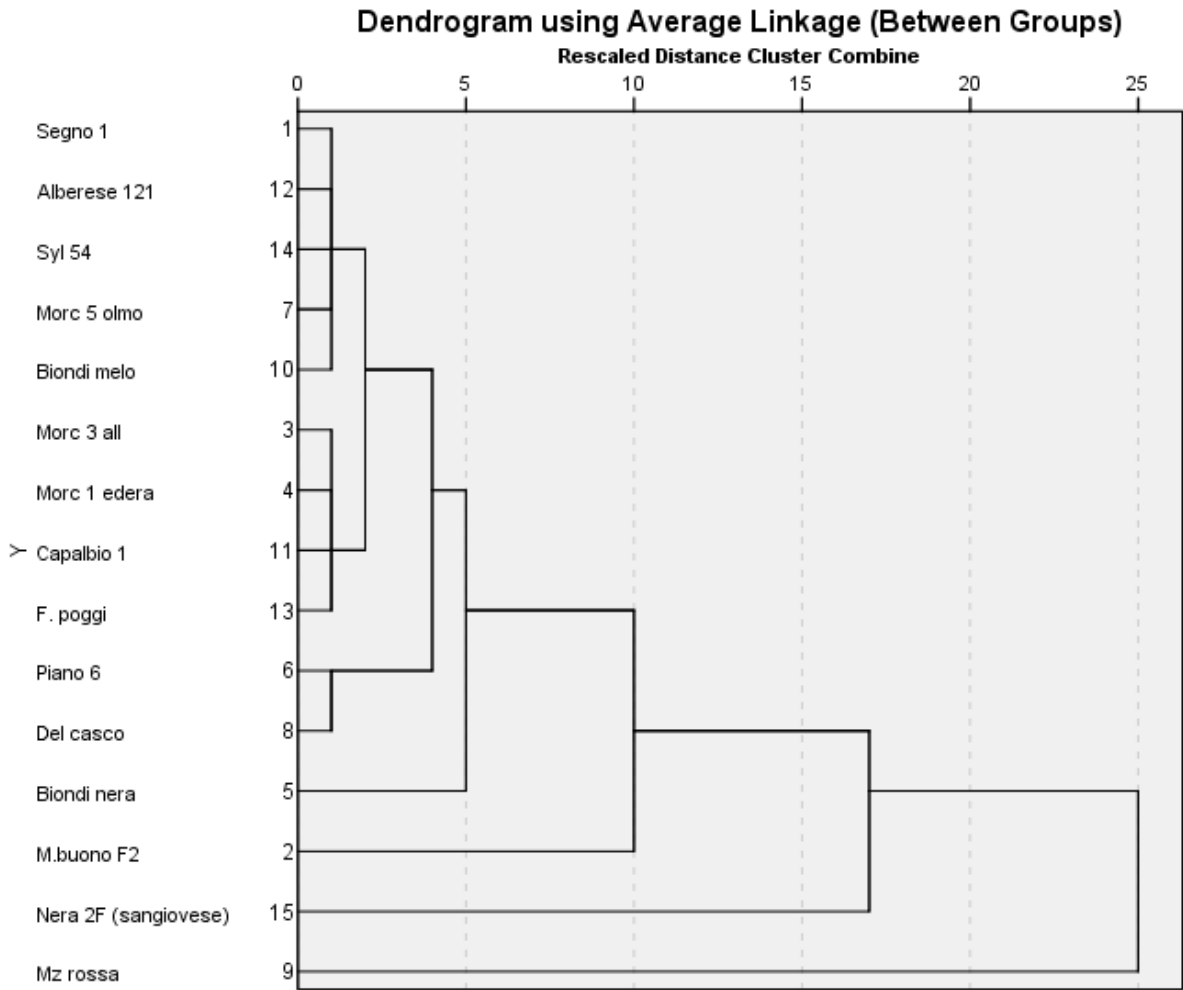
Figure 24: Statistical analysis of the phenolic richness.
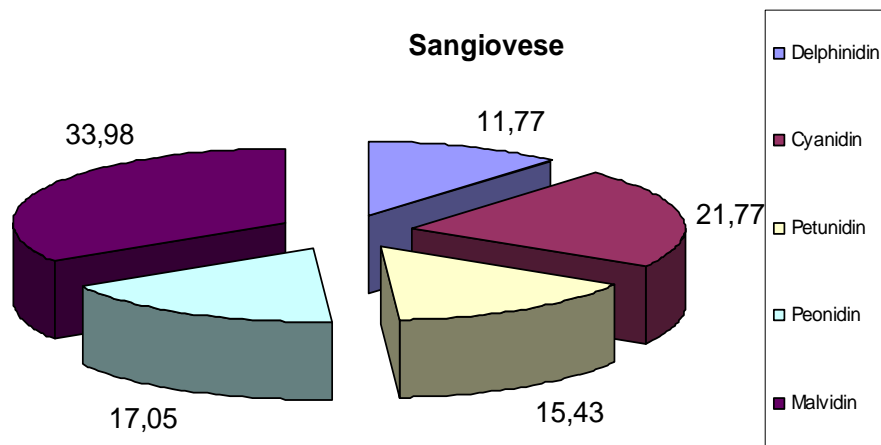
Figure 25: Anthocyanins profile of the cv. Sangiovese.
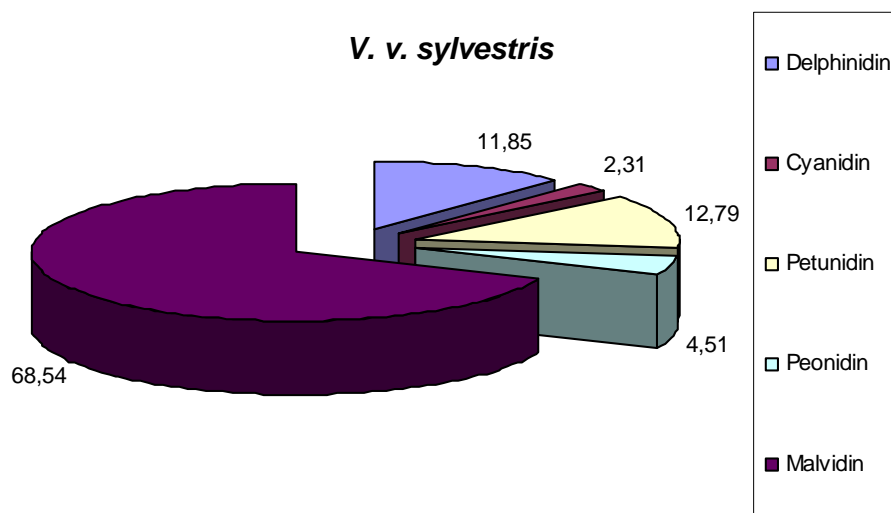
Figure 26: Anthocyanins profile of the *V. v. sylvestris* more representative.

Anthocyanins profile of some *V. v. sylvestris* (Tab. 18) were also compared with the profile of some cultivated varieties (Tab. 19), to evaluate possible similarity.

Table 18: Anthocyanins profile of some *V. v. sylvestris'* accessions.

Anthocyanins	37	48	51	52	59	60	68	69
Delphinin	23,63	13,77	11,85	19,36	14,78	8,14	4,63	18,27
Cyanin	5,43	1,67	2,31	2,54	9,66	4,47	20,32	2,68
Petunin	17,06	16,6	12,79	16,91	17,79	9,59	6,8	14,83
Peonin	8,53	4,73	4,51	5,19	21,08	30,05	44,86	6,55
Malvin	39,71	63,1	49,47	43,65	34,78	46,92	20,15	41,42
Acetate delph.	0,69	0,00	0,61	0,37	0,00	0,00	0,00	1,79
Acetate cyan.	0,15	0,00	0,31	0,19	0,00	0,00	0,50	0,39
Acetate petun.	0,37	0,00	1,77	0,6	0,00	0,00	0,28	1,60
Acetate peon.	0,96	0,00	2,29	0,28	0,00	0,34	0,74	0,80
Acetate malv.	2,04	0,00	5,70	2,61	0,42	0,49	0,02	5,55
P-cum. delph.	0,27	0,00	0,77	1,37	0,00	0,00	0,00	0,52
Caff. malv.	0,16	0,00	0,32	0,28	0,00	0,00	0,31	0,49
P-cum. cyan.	0,00	0,00	0,00	0,20	0,00	0,00	0,23	0,36
P-cum. petun.	0,00	0,00	1,29	1,39	0,00	0,00	0,00	0,50
P-cum. peon.	0,11	0,00	0,79	0,63	0,34	0,00	0,57	0,67
P-cum. malv.	0,88	0,13	5,21	4,42	1,14	0,00	0,58	3,57
Anhtocyan. 3 MG	94,36	99,87	80,93	87,65	98,09	99,17	96,76	83,75
Anthocyan. acetate	4,21	0,00	10,68	4,05	0,42	0,83	1,54	10,13
Anthocyan. p-cum.	1,26	0,13	8,06	8,01	1,48	0,00	1,38	5,62
Acet./p-cum.	3,34	0,00	1,33	0,51	0,28		1,11	1,80
Anthocyan. disubst.	15,18	6,40	10,21	9,03	31,08	34,86	67,22	11,45
Anthocyan. trisubst.	84,81	93,60	89,78	90,96	68,91	65,14	32,77	88,54
Trisubst./Disubst.	5,59	14,63	8,79	10,07	2,22	1,87	0,49	7,73

Some accessions show low quantity of acetate anthocyanins (48, 59, 60, 68) according to the Spanish accessions of *V. v. sylvestris* (Revilla *et al.,* 2012). While in others (69, 51), these values are more high (10.13 and 10.68).

Accessions 51 and 52 show high level of p-cumarate anthocyanins (8.06; 8.01) to the 69 (5.62) while 37, 48, 59 had very low content and in 60 was absent.

Another large different was found in the ratio trisubst./disubst which was very low on 68, 60, 59 (from 0.49 to 2.22). While the 48 shoe the higher value (14.63).

Table 19: Anthocyanins profile of some *V. v. Vinifera.*

Anthocyanins	Colorino	Tempranillo	Ciliegiolo	Giacomino	Sangiovese
Delphinin	6,17	14,64	9,6	3,48	11,77
Cyanin	1,01	1,8	6,51	2,25	21,77
Petunin	7,84	12,09	10,33	4,22	14,43
Peonin	8,64	4,37	18,7	13,08	17,05
Malvin	57,81	44,28	51,28	66,13	33,89
Acetate delph.	0,96	0,94	0,16	0,00	0,00
Acetate cyan.	0,42	0,00	0,00	0,00	0,00
Acetate petun.	1,14	0,49	0,14	0,00	0,00
Acetate peon.	1,70	1,74	0,13	1,12	0,00
Acetate malv.	7,00	2,95	0,56	3,94	0,00
P-cum. delph.	0,44	0,43	0,09	0,00	0,00
Caff. malv.	0,71	0,36	0,14	0,59	0,38
P-cum. cyan.	0,28	0,00	0,00	0,00	0,12
P-cum. petun.	0,74	2,40	0,14	0,78	0,00
P-cum. peon.	0,78	0,82	0,60	0,96	0,17
P-cum. malv.	4,36	12,68	1,62	3,45	0,42
Anhtocyan. 3 MG	81,47	77,18	96,42	89,16	98,91
Anthocyan. acetate	11,22	6,12	0,99	5,06	0,00
Anthocyan. p-cum.	6,60	16,33	2,45	5,19	0,71
Acet./p-cum.	1,70	0,37	0,40	0,97	0,00
Anthocyan. disubst.	12,83	8,73	25,94	17,41	39,11
Anthocyan. trisubst.	87,17	91,26	74,06	82,59	60,89
Trisubst./Disubst.	6,79	10,45	2,86	4,74	1,56

As the anthocyanins profile would be very important for the final wine quality and the color stability it is interesting to note differences and similarity between the accessions previous analyzed and five varieties cultivated in Tuscany. First of all, the cultivars more provided of malvin are Giacomino, Colorino and Ciliegiolo (respectively percentage of: 66.13; 57.81; 51.28), while between the wild types there was a larger variability of malvin content between a minimum of 20.15 % on 68 and the maximum of 63.1 % on 48.

In addition, the cvs. Sangiovese and Ciliegiolo have a low quantity of both acetate and p-cumarate anthocyanins, which is a similar pattern to what found in accession number 60.

The cvs. Tempranillo and Colorino have the major level of acetate and p-cumarate anthocyanins, while between the wild types we found comparable levels on 51 and partially on 52 and 69.

Analysis of the parentage of wild individuals also revealed relationships between nearby wild individuals, but in some case, analysis revealed pollen immigration from vineyards, confirming the fitness of the hybrid seedlings (Di Vecchi *et al.,* 2009).

Then, some grape were subjected to micro-vinification and the wine obtained were chemically analyzed (Tab. 20).

Table 20: Chemical analysis of the wine.

Accession	Alcohol (%Vol.)	Sugar (g/L)	Total Ac. (g/L)	Vol. acidity (g/L)	pH	Dry extract	Total Antoc. (mg/Kg)	Total Polyphenols (mg/Kg)
F. poggi	10,93	1,1	7,32	0,20	3,67	34,36	1022	4138
Mz rossa	10,46	0,8	7,19	0,20	3,83	37,44	984	3649
Del casco	8,23	1,9	7,45	0,20	3,50	32,27	1072	2713
M. buono F2	12,52	0,3	5,54	0,41	4,22	43,40	1421	4952
Morcola 1 edera	13,03	2,4	6,95	0,05	3,21	33,80	659	1854
Morcola 3 alloro	13,00	2,2	6,01	0,14	3,43	33,59	301	1296
Piano 6	9,66	1,9	7,13	0,28	3,26	32,41	614	2090
Nera 2F	12,82	1,5	4,05	0,31	3,55	25,32	298	1509
Morcola 2 giov	12,51	0,7	6,24	0,23	3,39	27,06	-	-
Mz bianca	13,91	2,3	5,28	0,50	3,61	27,00	-	-

The results highlighted some interesting aspect: good alcohol content for the white wine (the last two in the table above); high value of total acidity; very good dry extract; particular phenolic richness respect to the control.

3.6 Molecular characterization

A first analysis of microsatellite profiles some accessions revealed some cases of synonymy. For wild vines recovered in Tuscany was found one case of genetic similarity between the accessions "Biondi 2" and "Biondi 3" (Fig. 27), while all the others were appeared genetically different from each other, even if coming from the same area. It was also compared these microsatellite profiles with those of three, such as: Sangiovese, Buonamico and Colorino. First dominated in the past and present history of viticulture in Tuscany. For the cv. Buonamico and Colorino was shown in other publications (Di Vecchi *et al.,* 2006) to have a significant genetic similarity with the wild vines. In our case, no accession showed allelic profiles similar to any of the three varieties and the genetic diversity of wild grapevine populations was similar than that observed in the cultivated group.

The nuclear microsatellites analysis has shown that certain supposed accessions of *Vitis vinifera* subsp. *sylvestris* retrieved in Tuscany seem to derive from the vines already cultivated that become wild, while others accessions would be intraspecific cross-breedings *sativa-sylvestris*.

So, 22 accessions are already provided of all description and pictures, and are ready to be inserted in the Italian Vitis Database (www.vitisdb.it).
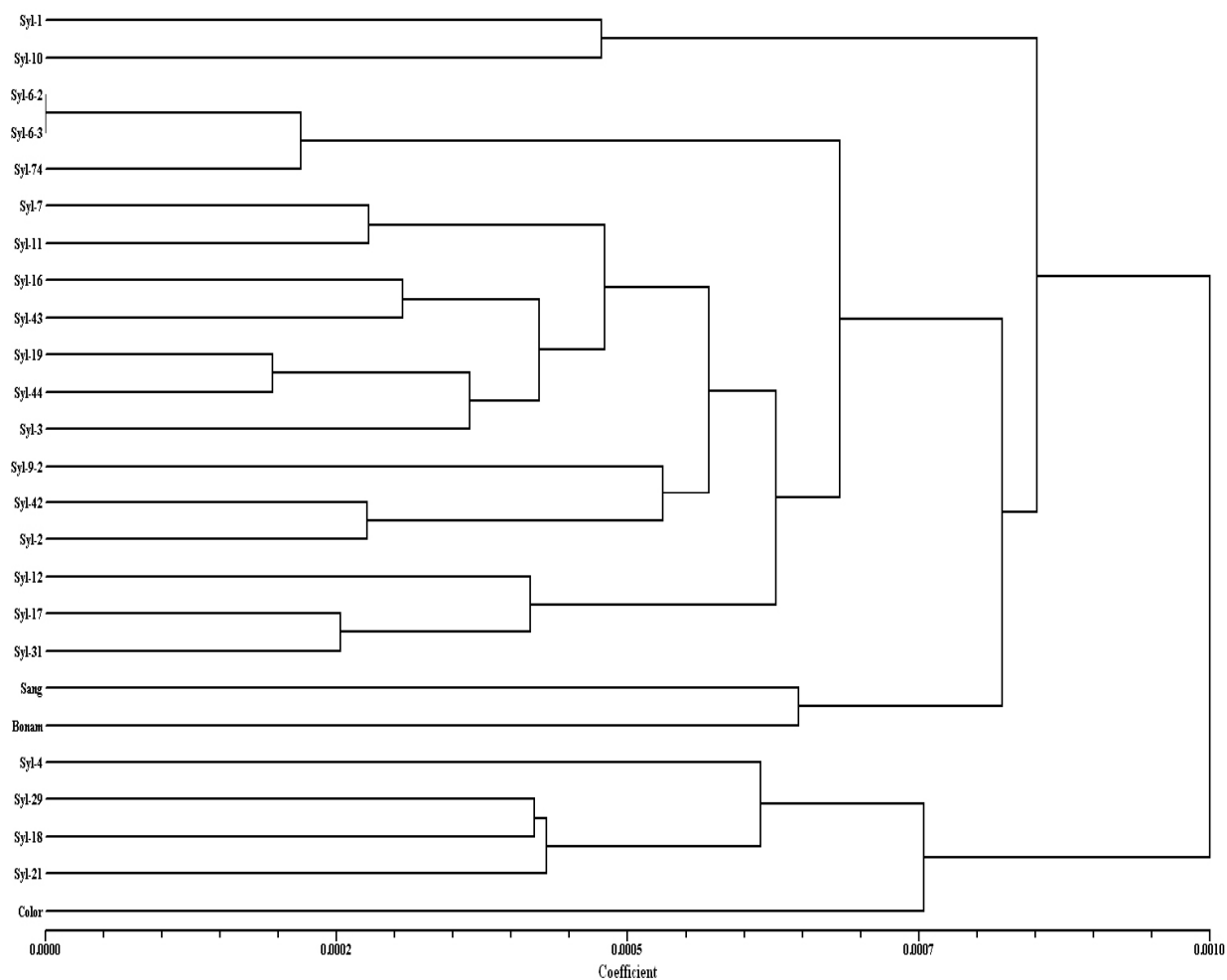
Figure 27: Genetic similarity between some *V. v. sylvestris* and some cultivated varieties.

Genetic relatedness of cultivars has been shaped mostly by human uses, in combination with a geographical effect. The finding of a large portion of admixed genotypes may be the trace of both large human-mediated exchanges between grape-growing regions throughout history and recent breeding (Bacilieri *et al.,* 2013).

3.7 Search of new accessions

The search of new biotypes of *V. v. sylvestris* allowed us to find 31 new accessions (Tab. 21) with a total of 175 plants that now are in pots of 1 L capacity everyone, ready to be planted.

Table 21: Lists of the accessions in plots.

	Accession	Origin	Sex	Color
1	11 dopo ponte	Alberese	F	B
2	13 conte A	Alberese	?	?
3	12 prima del ponte	Alberese	?	?
4	Syl 22	Alberese	M	-
5	Syl 59	Alberese	?	?
6	Alberese OF 1M	Alberese	M	-
7	Alberese OF 2F	Alberese	F	B
8	Vigili	Borgo S. Rita	?	?
9	Syl 130	Manciano	?	?
10	Syl 1 2014	Manciano	?	?
11	Syl 3 2014	Manciano	?	?
12	Syl 4 2014	Manciano	?	?
13	Syl 5 2014	Manciano	?	?
14	Syl 7 2014	Manciano	?	?
15	Syl 8 2014	Manciano	?	?
16	Syl 9 2014	Manciano	?	?
17	Syl 10 2014	Manciano	?	?
18	Syl 11 2014	Manciano	?	?
19	Centro aziendale	Poggi del Sasso	?	?
20	Syl 116	Sasso d'Ombrone	?	?
21	Syl 115	Sasso d'Ombrone	?	?
22	Syl 61	Sasso d'Ombrone	F	B
23	Syl 110	Sasso d'Ombrone	?	?
24	Syl 118	Sasso d'Ombrone	?	?
25	Syl 76	Sorano	F	B
26	Syl 21 2014	Sovana	?	?
27	Syl 22 2014	Sovana	?	?
28	Syl 23 2014	Sovana	?	?
29	Syl 24 2014	Sovana	?	?
30	Syl 25 2014	Sovana	?	?
31	Syl 26 2014	Sovana	?	?

Moreover, other 33 accessions (Tab. 22), grown in very difficult environmental condition, were found. Due to their position (Fig. 28), which make impossible to take plant material for hardwood propagation, these biotypes were only marked in their natural habitat.

Table 22: List of those accessions grown in difficult environment which were only marked.

	Accession	Origin	Sex	Color
1	1	Alberese	F	W
2	50	Alberese	F	B
3	2	Alberese	F	W
4	4	Alberese	F	B
5	14	Alberese	?	?
6	17	Alberese	F	?
7	20	Alberese	?	?
8	21	Alberese	?	?
9	26	Alberese	?	?
10	27	Alberese	?	?
11	28	Alberese	?	?
12	29	Alberese	?	?
13	30	Alberese	?	?
14	30 A	Alberese	?	?
15	30 B	Alberese	?	?
16	30 C	Alberese	?	?
17	31	Alberese	F	?
18	32	Alberese	?	?
19	33	Alberese	?	?
20	34	Alberese	?	?
21	38	Alberese	?	?
22	39	Alberese	F	?
23	40	Alberese	?	?
24	41	Alberese	?	?
25	42	Alberese	?	?
26	43	Alberese	F	?
27	44	Alberese	?	?
28	45	Alberese	F	?
29	46	Alberese	?	?
30	49	Alberese	F	?
31	51	Alberese	?	?
32	52	Alberese	?	?
33	55	Alberese	F	?

Figure 28: Plants grown in inaccessible area.

4 CONCLUSIONS

From ampelographyc and ampelometric point of view the accessions differ quite significantly between them about their area of origin, presenting morphological characteristics more or less homogeneous and distinguishable between different habitats. Probably, they followed an evolutionary line that changed their phenology in a similar way.

These plants are quite susceptible to mildew infection although less or equal proportionally to the cv. Sangiovese used as control.

About virus presence we found a good sanitary condition even though only few accessions were infected by four of the nine different viruses investigated, in separately or associated way. They were probably infected in their area of origin, because were near to commercial vineyards retrieved.

Most of the accessions recovered had bud-break fairly early or relatively intermediate, as they approached very much to the behaviour of cv. Sangiovese, which is an early variety. Studies of end of dormancy and temperature requirement revealed differences on the behaviour and response to winter and forcing temperature on some representative accessions, suggesting genetic influence. In fact, the accessions 48, 26 and 53 showed higher GDH requirement in the field conditions than the forcing chamber, while other accession had a lower GDH requirement in field conditions exhibiting an earlier bud-break.

Most of the accessions studied (black and white) reached at harvest a sufficient technological maturity and were characterized by very small berry and clusters, in agreement with the literature on this topic.

In addition, quite marked differences on polyphenol content and especially in the anthocyanin profile between *V. v. sylvestris* grapes and the reference (Sangiovese) variety were found. The concentration of anthocyanins in the skins, in some cases resulted much higher, also *V. v. sylvestris* berries had a larger prevalence of malvidin and a low amount of cyanine and peonin.

Wines made from micro-vinification, are particularly rich in tartaric acid, full bodied and rich in anthocyanins, even if they are unbalanced.

From molecular characterization only two accessions were equal (case of synonymy), while all the others were different regardless their geographic origin. Also, no one showed allelic profile similar to the varieties cultivated in Tuscany with which they were compared.

The peculiarities of some accessions (phenolic richness and anthocyanin profile) could be useful for further grapevine breeding programs.

The discovery of 64 new accessions and the large area that still could be explored, especially within the "Parco Naturale dell'Uccellina" (Alberese – GR), confirms that the Tuscany Maremma is very rich of *V. v. sylvestris* and that their genetic pattern is quite differentiated, so as these patrimony should be preserved by genetic erosion. In conclusion, the data obtained in this study reinforce the need to protect these rare and valuable genetic resources and pointed out that further studies are necessary to show how in the domestication process of the cultivated grapevine, could have taken place an introgression from Western wild forms of *Vitis vinifera* in the pedigree of some current Western European cultivars.

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