Plant immunity as a result of co – evolution

Using the pair grapevine / downy mildew as a model

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Zusammenfassung

Das Pathosystem *Vitis* (Wein) / *Plasmopara viticola* (falscher Mehltau) ist in vielerlei Hinsicht hoch interessant. Zum einen ist *P. viticola* genetisch nicht eindeutig bestimmt, und vermutete Subspezies konnten noch nicht eindeutig nachgewiesen werden. Zum anderen wurde der Erreger vor 150 Jahren von Amerika, wo er mit resistenten *Vitis* Spezies eine Co-Evolution durchlief, nach Europa eingeschleppt, wo er auf die naive – und deshalb empfängliche – europäische Kulturrebe *Vitis vinifera*, sowie auf die europäische Wildrebe *Vitis sylvestris*, von der bisher keine genauen Angaben über etwaige Resistenzen vorlagen, stieß.

Ein Ziel dieser Arbeit war es nun, die verwandtschaftlichen Verhältnisse verschiedener *P. viticola* Isolate aus Europa und Amerika aufzuklären. Unter zu Hilfenahme verschiedener DNA-Sequenzen aus dem Zellkern und den Mitochondrien wurden daraus Stammbäume erstellt. Zudem sollte durch die Erhebung physiologischer und morphologischer Daten ein Zusammenhang zwischen *P. viticola* resistenten und nicht resistenten *Vitis* Arten hergestellt werden.

Ein weiteres Ziel war, anhand einer stabilen *Vitis sylvestris* Population von der Rheininsel „Ketsch“ herauszufinden, in wieweit Introgression stattfindet und ob eine Präadaption gegenüber *P. viticola* vorliegt.

Im Rahmen dieser Arbeit konnte gezeigt werden, dass weitere *P. viticola* Stämme existieren, diese aber nicht in Europa vorkommen und zudem keine Veränderung im Infektionsverhalten gegenüber *Vitis* aufweisen. Es konnte auch gezeigt werden, dass die Zoosporen von *P. viticola* die Stomata der Pflanzen mit Hilfe eines Nonanal Gradienten aufspüren. Einige Reben Spezies emittieren allerdings weniger Nonanal als andere und können deshalb nicht so leicht infiziert werden. In Anwesenheit von exogenem Nonanal wurde die Infektionsrate bei der Kultursorte „Müller-Thurgau“ um durchschnittlich 75% reduziert.

Weiterhin konnte gezeigt werden, dass die Population der europäischen Wildrebe im Verbreitungsgebiet „Ketsch“ einerseits durch geringen Genfluss frei von Eintragung fremder Pollen wie zum Beispiel die der Kulturrebe oder die von Rebunterlagen ist, und andererseits durch eine ausreichende genetische

Diversität vor Inzucht geschützt ist. Anschließend konnte gezeigt werden, dass die europäische Wildrebe *Vitis sylvestris* durch Präadaption resistent gegen den falschen Mehltau ist.

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1. Introduction

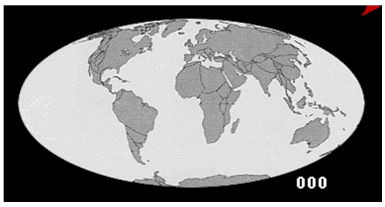
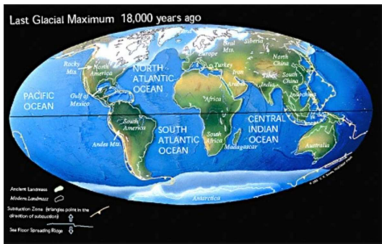
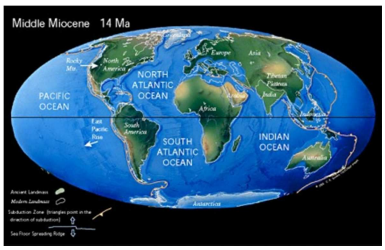
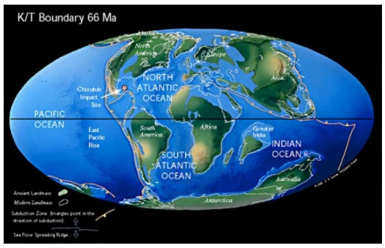
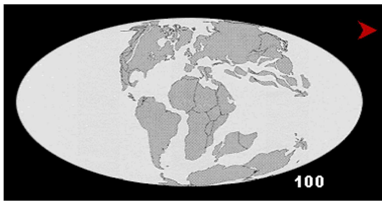
Plasmopara viticola, the grapevine false downy mildew, is an oomycete, and it is the most important grapevine pathogen. It was introduced to Europe from North America in the last third of the 19th Century. Since then, *P. viticola* has spread all over Europe and caused great economic damage in vineyards due to yield and quality losses. Depending on the development and severity of the epidemic, more than a quarter of the yield of crop production might be destroyed, but also total losses are possible. Due to the high value of the product “wine”, profits up to 40.000,- € and even more than 50.000,- € per hectare can be expected per year. Therefore, a reduction of 25% in crop yield could lead to a financial loss of at least 10.000,- € per hectare per year. In Germany, the traditional cultured grapevine varieties are highly susceptible to *P. viticola*. Therefore, profitable quality grape growing without the use of synthetic chemical pesticides is not possible. More than 100.000 hectares of vineyards in Germany undergo 6-9 fungicide treatments against downy mildew per year. This is not only a high financial burden on the grape growers, but also introduces about 175 tons of plant protection products into the ecosystem every year. A primary goal of the German grapevine industry is, therefore, to ensure quality and yield of crop production with a more environmental friendly method to control *P. viticola* infection. An alternative approach to control the disease, without using synthetic chemical pesticides, is the introgression of wild species from North America, which have co-evolved with the pathogen and therefore are rather resistant to infection. Another approach would be the activation of the plant's own immune system leading to a defense response against the pathogen.

1.1. Geographic distribution of Vitis

1.1.1. History of the evolution of Vitaceae

The family of Vitaceae developed most likely during the late Cretaceous or early Tertiary era in subtropical regions of what is now continental Africa (Tab. 1). Findings from South West Africa give evidence of simplest types of stem structure (i.e., pure Monopodia, spiral phyllotaxis, succulent Cissus species), and plants lacking twine growth. More than 300 fossil sites reveal evidence of Vitaceae, however, much of these records might be misidentified, as the findings were not well preserved. For example, several findings of *Vitis* from the Cretaceous era in Wyoming, USA are ambiguous (Engler & Prantl, 1953). Leaf and wood findings were considerd in particularly to be deceptive, for identification purposes. However, findings of seeds can be considered as reliable, as exampled for the genera *Ampelocissites*, *Ampelocissus*, *Ampelopsis*, *Cayratia*, *Cissus*, *Palaeovitis*, *Tetrastigma* and *Vitis*, which all date from the Tertiary era. To date, the oldest cogent findings originate from southeast England (*Ampelopsis*, *Cayratia*, *Palaeovitis*, *Tetrastigma* and *Vitis*) and South-Eastern North America (*Ampelocissites*), dating back to the early Eocene. Findings from late Eocene were located in Peru (genera *Cissus* and *Ampelocissus*), and on the South coast of England (*Ampelopsis*, *Parthenocissus*, *Tetrastigma* and *Vitis*). Additionally, the genus *Vitis* was also found in the Arctic. Findings of *Vitis* from the Oligocene and Miocene eras were located in Europe (*Vitis teutonica*, *Vitis sylvestris* and *Vitis parasylvestris*). In the Pliocene era, remains of *Vitis* such as *V. ludwigii* were found in Europe, North America and Japan. Whether these species are already identical to the recent taxa, is not yet clear (Engler & Prantl, 1953).

Today, the majority of *Vitis* species (e.g. *V. rupestris*) are located in North America, and slightly fewer species (e.g. *V. amurensis*) in Asia. In Europe, however, only *V. vinifera (ssp. sylvestris)* is found, although spread over a vast area.

Cenozoic - Quaternary	**Holocene** 10kya till present	
	Pleistocene 1.6-0.01 Mya	
Cenozoic - Tertiary 66.4-1.6 Mya	**Pliocene** 5-2 Mya	
	Miocene 25-5 Mya	
	Oligocene 38-25 Mya	
	Eocene 55-38 Mya	
	Paleocene 65-55 Mya	
Mesozoic 245-66.4 Mya	**Cretaceous** 144-66.4 Mya	

Tab. 1: Geological timetable (Mya = Million years).

1.1.2. Distribution of *Vitis vinifera* ssp. *sylvestris* (C. C. Gmel.) Hegi and *Vitis vinifera* ssp. *vinifera* L.

Dispersion of berries, probably by birds, allowed *Vitis vinifera* ssp. *sylvestris* (in the following designated as *V. sylvestris*) to spread over broad areas. The current distribution is considered to be a relict of the Tertiary flora of which wild grape plants were widespread across southern and parts of central Europe. *Vitis sylvestris*-like seeds, also known as *V. parasylvestris* or *V. sylvestris* Gmelin, were recovered from sites of the Oligocene and subsequent periods, and disseminated to the latest Pliocene. However, from the beginning of the first glaciation this species had disappeared from Europe and is not detectable in finds from the Pleistocene era, neither in the icy Northern region nor in the Alpine glaciers. However, *V. sylvestris* persisted in Southern France and Italy. The present *V. sylvestris* species probably derived from these glacial relicts (Engler & Prantl, 1953). Today, *V. sylvestris* only exist in few locations in the Upper Rhine valley (Germany) and in the Danube floodplain (Lower Austria and Moravia).

According to archeological evidence (Olmo, 1996), the domestication of *V. sylvestris* and its conversion into the efficient high yield and quality plant *Vitis vinifera* has been traced to Transcaucasia. However, phylogenetic analysis of the two subspecies ssp. *sylvestris* (C. C. Gmel.) Hegi and ssp. *vinifera* L., based on plastidic markers, suggest at least two origins of cultivated grapevines in the Middle East, and in the Iberian Peninsula (Arroya-García *et al.*, 2006).

1.2. Characterization of the pathogen: *Plasmopara viticola*, false downy mildew

1.2.1. Taxonomy

Plasmopara viticola belongs to the group of oomycota, which consists of a single class, the oomycetes (Tab. 2). Up to several years ago, the oomycetes were still classified as fungi. However, according to sequence comparisons of small subunit rRNA experiments (Gunderson *et al.*, 1987), it was revealed that they actually belong to the kingdom of the Chromista (Gams *et al.*, 1988; Unger, 2001) together with chloroplast bearing algae, containing chlorophyll a and c (e.g. diatoms), or colorless algae. As additional characteristics the zoospores of the Chromista are endowed with heterokont flagella (Alexopoulo *et al.*, 1996). In contrast to the true fungi, the cell wall of oomycetes consists of cellulose and does not contain chitin (Bartnicki-Garcia, 1968).

class Oomycetes
order Saprolegniales
order Peronosporales
family Phytiaceae
family Peronophytoraceae
family Abuginaceae
family Peronosporaceae
genus *Basidiophora* (Roze et Cornu 1869)
Bremia (Regel 1843)
Parasperonospora (Constantinesu 1989)
Peronosclerospora (C.G. Shaw 1978)
Peronospora (Corda 1837)
Plasmopara (Schröter 1886)
P. viticola (Berk. et Curtis) Berlese et deToni
Pseudoperonospora (Rostovcer 1903)
Sclerophthora (C.G. Shaw 1953)
Sclerospora (Schröter 1879)
order Leptomitales
order Lagendiales

Tab. 2: Classification of Oomycota (according to Kortekamp, 1996).

1.2.2. Co-evolution of *Vitis* and *Plasmopara viticola* as well as development of pest control

The cultivated grapevine, *Vitis vinifera* represents one of the most economically important fruit crops worldwide. *V. vinifera* has been cultivated for at least 6000 years, starting in Transcaucasia / Mesopotamia (Olmo, 1996). The grape growing region expanded over the centuries to the European continent. However, in the 19th Century, the invasion of *Phylloxera* parasites (*Daktulosphaira vitifoliae*) devastated European vineyards. This native North American aphid was introduced to France in the mid-19th century (discovered in the 1860s) and spread rapidly all over European wine-growing regions. To control this pest, the susceptible scions of cultivated grapes were grafted on *Phylloxera*-resistant rootstocks derived from North America wild *Vitis* species, such as *Vitis riparia* and *Vitis berlandieri* in local European nurseries. As a consequence, the complicated reproductive cycle of *Phylloxera* was interrupted.

Unfortunately, this process of grafting also introduced a new disease to Europe, the false downy mildew, *Plasmopara viticola*. This disease first erupted in Europe in 1875 (Alexopoulos, 1966). In contrast to the American *Vitis* species, which have co-evolved with the pathogen and developed a resistance against it, the European cultivars were susceptible. A search began in order to develop a treatment preventing such infection. In 1885, Professor Alexis Millardet found a mixture of $Ca(OH)_2$ and $CuSO_4$, known as Bordeaux-broth (named after the university of its inventor) to be a reliable contact poison against zoospores of *P. viticola* due to the fact that it contains Cu^{2+} ions. Cu^{2+} ions act as enzyme toxins in the fungal spores, and prevent their germination. To date, the Bordeaux-broth is one of the most effective, but environmentally very problematic instruments in pest control as it kills micro-organisms and earthworms in the soil and also pollutes the groundwater.

Since then, several fungicides, such as Dithiocabarmates, which also acts as a contact toxin targeted to metal-containing enzymes (e.g. Polyphenoloxidases), and sulfur-containing enzymes (e.g. Glucose-6-phosphate dehydrogenase) were developed. In parallel, the natural resistance of North American wild *Vitis* species was introduced into cultivated grapevine by classical breeding

strategies. The first resistant grapevine variety (“Merzling”) from Seyve-Villard, Riesling and Pinot Gris, was developed in 1960 by Dr. J. Zimmerman at the State Institute of Viticulture in Freiburg, Germany. Recently, genetic engineering has been used to introduce new resistance genes into susceptible plants, for example by infection of the plants with genetically modified *Agrobacterium tumefaciens* (Bornhoff *et al.*, 2005). Despite the undoubted success of resistance breeding, latterly, grape growers report, that *Plasmopara viticola* apparently has further evolved and is able to affect even resistant grapevine varieties in distinctive areas demonstrating that efficient resistance management is needed to secure the sustainability of this strategy.

1.2.3. Symptoms of pathogen infection

Plasmopara infections are evident at the leaf surface as smooth, yellow brightened, oily shiny spots, so-called oil spots (Fig. 1). In humid weather conditions, the pathogen sporulates on the lower leaf surface, and it forms a dense, white mycelium (Fig. 2). When the infection is older, leaves had shown also necrotic lesions. The infections sites remain small and are limited by the surrounding leaf veins (Fig. 2). Strongly infested leaves turn brown and fall off. Similarly to leaves, *Plasmopara viticola* also infects clusters (young or mature berries), green shoots and leaf stems. During the infection process, a mycelium is formed in the mesophyll, which becomes manifest at the surface as an oil spot. Eventually, the infected plant tissues show brown necrosis similarly to the infection on the leaves. In big-sized berries (diameter > 0.8 cm), the fungus can grow into the interior of the berries. In this case, no white fungus lawn will develop, but the berries would change their color to blue violet, dry out and turn into so-called leather berries (Fig. 3). This damage to plant leafs and berry loss can lead to crop losses up to 50-70% (Agrios, 1997).

Fig. 1: Oilspot with necrotic lesion on top of the leaf (Gessler, 2005).

Fig. 2: Sporangia at the lower leaf surface (Gessler, 2005).

Fig. 3: Leather berries (Gessler, 2005).

1.2.4. Developmental cycle

The developmental cycle of *Plasmopara viticola* is divided into two phases, as shown in figure 4: In late fall and winter seasons, female sexual organs (oogonia) are formed in the fallen leaves after fertilization of an oospore (winter spore). The fungus overwinters in the fallen leaves. The winter spores are very thick-walled, and thus resistant to cold, wet and dry conditions. Protected by their thick cell wall, they may survive for many years. In spring, the primary infection is caused by those spores. Under suitable conditions, the disease can spread massively. The more humid the months of April and May, the earlier a primary *Plasmopara* infection is possible. The winter spores germinate only in damp weather, with rainfall of at least 10 mm and a 24-hour average temperature of more than 8° C.

At the end of the germ tube, an oval proliferation cell (primary sporangium) is formed (Fig. 4). When it rains, the spores or even the whole sporangium get splashed on the leaves. Infection to young shoots is possible in May, when the plants are more than 10 cm long. After bursting of the shell, the sporangium

releases up to 60 diploid spores (zoospores) (Unger, 2001). These flagellated zoospores reach to the stomata of the leaves using the water film on the leaf surface. There they encyst and germinate, causing an infection. The germ tube, which is then generated, penetrates into the respiratory cavity of the stoma. Once located there, a hypha with haustoria develops, which secures the nutrition of the fungus (Kortekamp *et al.*, 1998). After an incubation period of several days, the infection is already visible on the leaf as an "oil spot". The incubation period depends on the temperature and can take 4-18 days.

Such a primary infection is the base for further infections: In humid weather conditions, it only takes few days to produce a visible white coating of sporangiophores underneath the leaf. The sporangia containing the zoospores are spread by wind and rain. The visible white mycelium on the lower leaf surface carries thousands of such sporangia, which can be carried away even at a slight breeze over long distances. Once in touch with rain water or even dew drops, 5-8 zoospores can be released from a single sporangium. This leads to further infections. Thus, under appropriate conditions, the fungus multiplies vegetatively continuously until autumn, and the number of infection cycles depends on the annual weather conditions. It was shown, that under favorable conditions (humid and dark), the infection process can be repeated five to eight times a year (Schlösser, 1997).

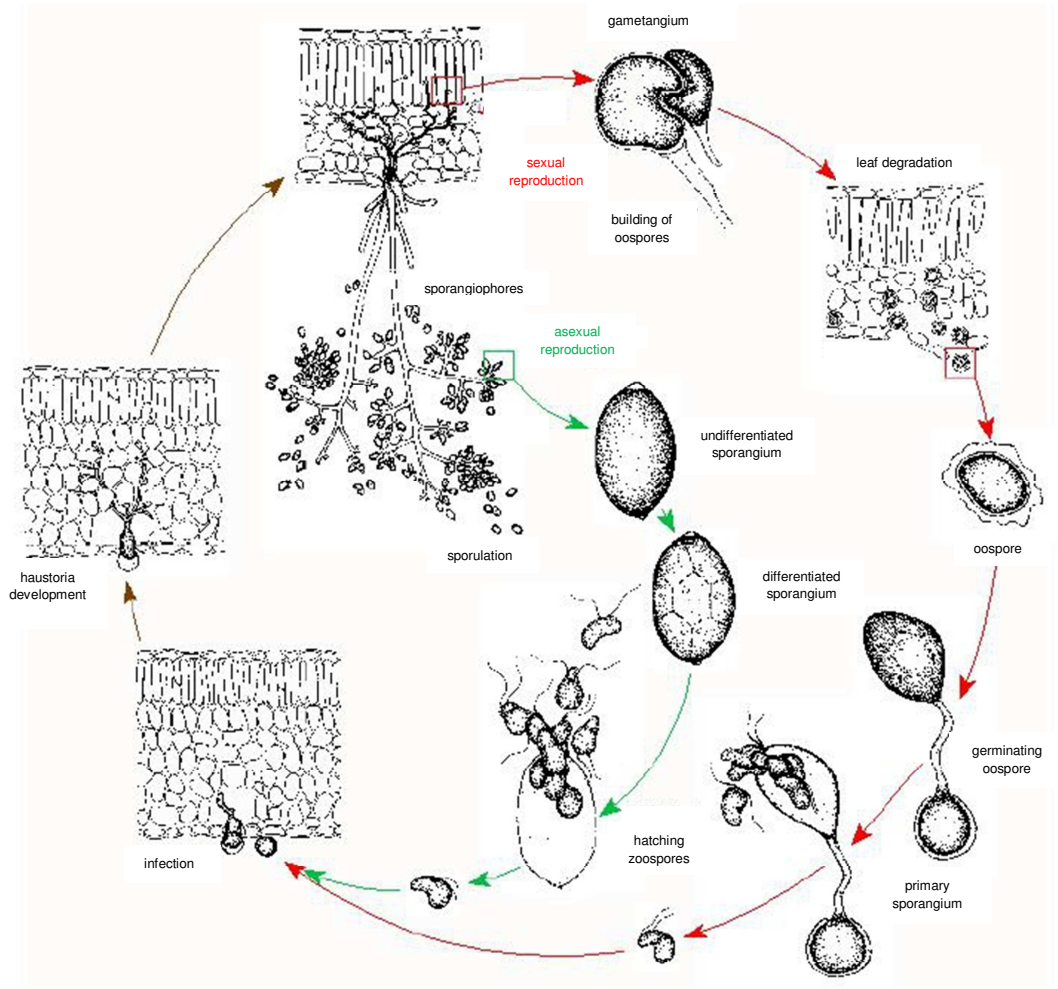


Fig. 4: Developmental cycle of *Plasmopara viticola*. Sexual cycle marked with red, asexual cycle with green arrows. Brown arrows mark the stages, which are shared by both cycles. Figure modified from: N. Schöpe, (Unger, 2001).

1.3. Mechanisms of plant defense against pathogen infection

Host and pathogen (or herbivores) always pass through a co-evolution over time. Whether it is a virus, that changes its protein shell, DNA or RNA sequence to be no longer recognized as a pathogen for the host, or a unicellular organism that obtains new abilities by mutation, or whether there are herbivores that are resistant to certain toxins, as a consequence of this co-evolution, infection strategies are adapted progressively to the respective host.

Plants, on the other hand, were forced to develop new defense mechanisms. Various defense mechanisms exist against plant pathogens in plants. The first one is prevention of infection by external barriers such as the formation of a cuticle, thickened or lignified cell walls or the formation of secondary metabolites such as phenols, stilbenes, or saponins (Unger, 2001). The second one is the immediate defensive response, the so-called “local response”. In some cases, a “hypersensitive response” involving gene activation and induction of programmed cell death is associated with the local response. Finally, in many cases, a systemic acquired resistance (SAR) response, which is also associated with an activation of genes, follows in the remaining parts of the plants that had no direct contact with the pathogen.

1.3.1. The immediate defensive response: PAMPs and the Gene-for-Gene concept in plant pathology – a host / parasite immunology co-evolution

Co-evolution is a central driving force of evolution itself. Predator-prey relationship and host-parasite systems are prime examples. Immunity, i.e. the ability of an organism to specifically detect and arrest an attack of an intruding organism, has to be understood in terms of co-evolution. Mammalian immunity has been studied in great detail due to its medical relevance. However, immunity of other organisms has not attracted the same degree of attention, although it has developed early in evolution. Even plants have an “immune system”, which reacts when they are infected by a pathogen. The immediate defence answer of a cell, or an infected part, consists in a local reaction: In the

extreme case, the, cells surrounding the infection site initiate a suicidal reaction (hypersensitive response) triggered by the formation of reactive oxygen species (O_2^-, •OH, H_2O_2) and nitrogen monoxide (NO) to avoid further spread of the infection. But also in those cells that do not undergo a hypersensitive response, so called phytoalexines (Greek: aleksein = to defend), i.e. secondary metabolites with anti-microbial activity (Heldt, 2003) are synthesized. The reactive oxygen species triggering hypersensitive responses and phytoalexin formation are activated by an NADPH-dependent oxidase in the plasma membrane, where O_2 is formed first. It is then demodulated into •OH and H_2O_2 (Taiz & Zeiger, 2000). The formation of nitric oxide (NO) can be synthesized by nitrite reductase or NO synthase from arginine and oxygen.

In the first step, the hypersensitive response is initiated by the innate immunity of the plant, triggered by general elicitors or so called PAMPs (pathogen-associated molecular pattern). This evolutionary ancient pathogen perception system recognises molecules that are characteristic for entire groups or classes of microorganisms (Zipfel & Felix; 2005). This innate plant immune system is mediated by different pattern recognition receptors (PRR), e.g. Toll- and Toll-like receptors (TLR) respectively, with extracellular LRRs (leucine-rich repeats), in analogy to other organisms, including mammals (Fig. 5). For example, it was possible to identify a flagellin receptor (FLS2) in Arabidopsis for the 22-amino-acid peptide flg22, which is a highly conserved component of bacterial flagella. When the peptide binds to the receptor, this will initiate a signal cascade of the MAP (mitogen activated protein kinase) kinase cascade type (involving the kinases MEKK, MKK4/MKK5 and MPK3/MPK6), that will induce early defence genes by activation of transcription factors WRKY22/29 (Fig. 5) (Asai *et al.*, 2002). There are also PAMPs characteristic for fungi and oomycetes (Zipfel, 2008). Some of these fungi- and oomycetes-derived patterns are recognized by only a few plant species whereas others, notably chitin, are recognized by all of the higher plant species (Zipfel & Felix, 2005).

A second layer of rapid cellular responses is triggered by a system described as “Gene-for-Gene model” (Flor, 1971). It is activated, when the PAMP-triggered immune (PTI) response was overcome by so called “effectors” of the pathogen causing an “effector-triggered susceptibility” (ETS) inactivating the PTI.

According to this model, the interaction between a given host cultivar and a specific strain of the phytopathogen is activated by specific elicitors that are released by the pathogen. These elicitors are protein-, lipid- and polysaccharid-fragments, secreted by the pathogen or leaking from its cell wall or external membrane (Boller, 1995). Elicitors are encoded by Avr- (avirulence-) genes and bind to receptors consisting of a characteristic nucleotide binding site (NB) and a leucine rich repeat (LLR) domain (Jones & Dangl, 2006) on the cell surface which are primarily formed by "R genes", i.e. plant-resistance genes (Taiz & Zeiger, 2000). The binding of an Avr-gene product (originating from the pathogen) to the R-gene product (originating from the host) triggers a signal cascade culminating in a hypersensitive response (“effector-triggered immunity”, ETI) (Fig.6) of the attacked host cell including a synthesis of "pathogenesis-related proteins" (PRPs) such as glucanases and chitinases which attack fungal cell walls (Hammond-Kosack & Jones, 1997). This reaction will only happen, when the plant “recognizes” the elicitor according to a key-lock principle.

Interestingly, the receptors initiating the recognition systems relevant for Avr-triggered, PAMP-triggered, and symbiosis-triggered responses are composed from modular domains that are combined to yield different functions (Fig. 7).

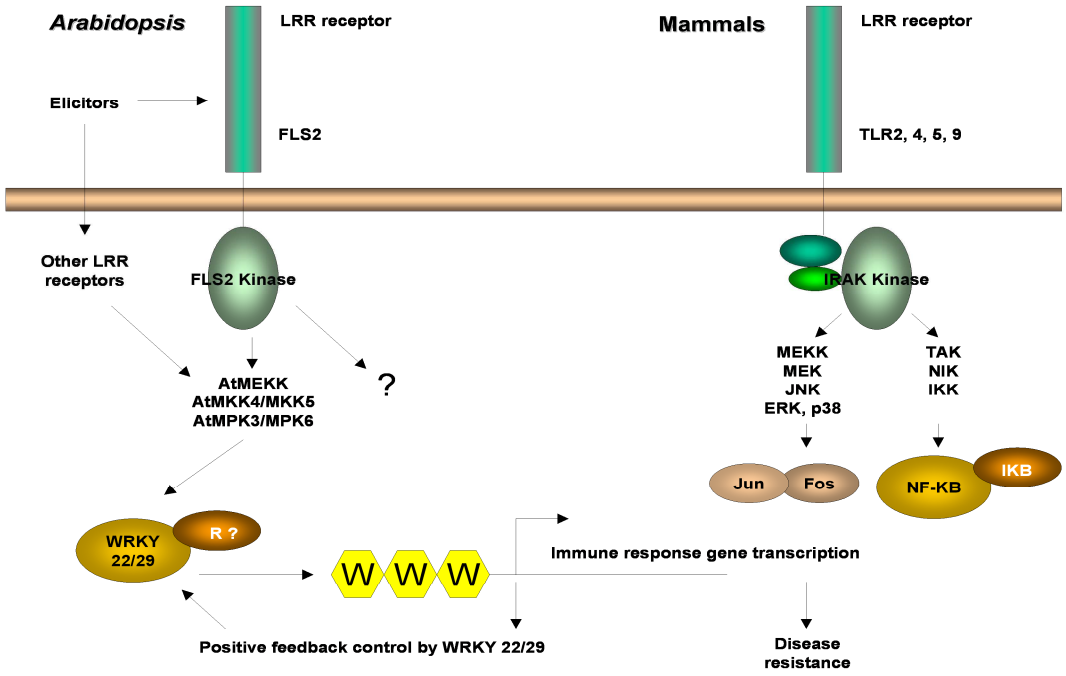


Fig. 5: Model of innate immune signalling activated by LRR receptors in *Arabidopsis* and mammals. Binding of a putative repressor (R) controling WRKY22, WRKY29 and WRKY42 activity to the W-box is modulated by the signaling (according to Asai *et al.*, 2002).

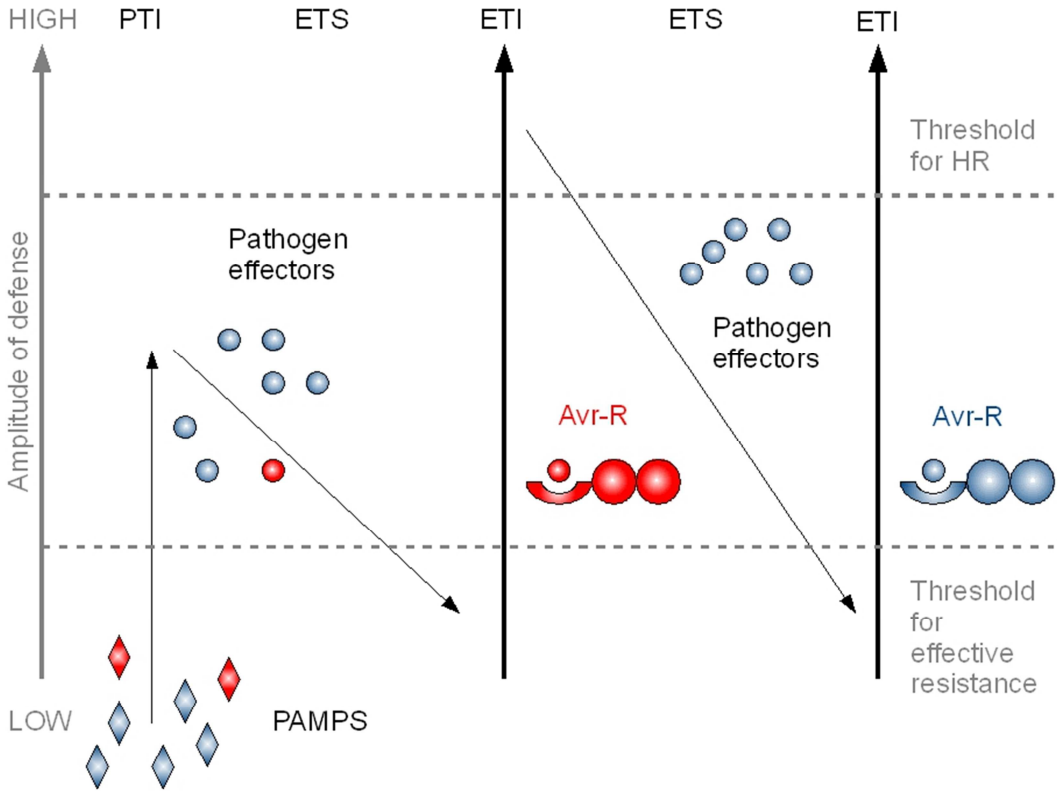


Fig. 6: Zigzag model of the quantitative output of the plant immune system (according to Jones & Dangl, 2006).

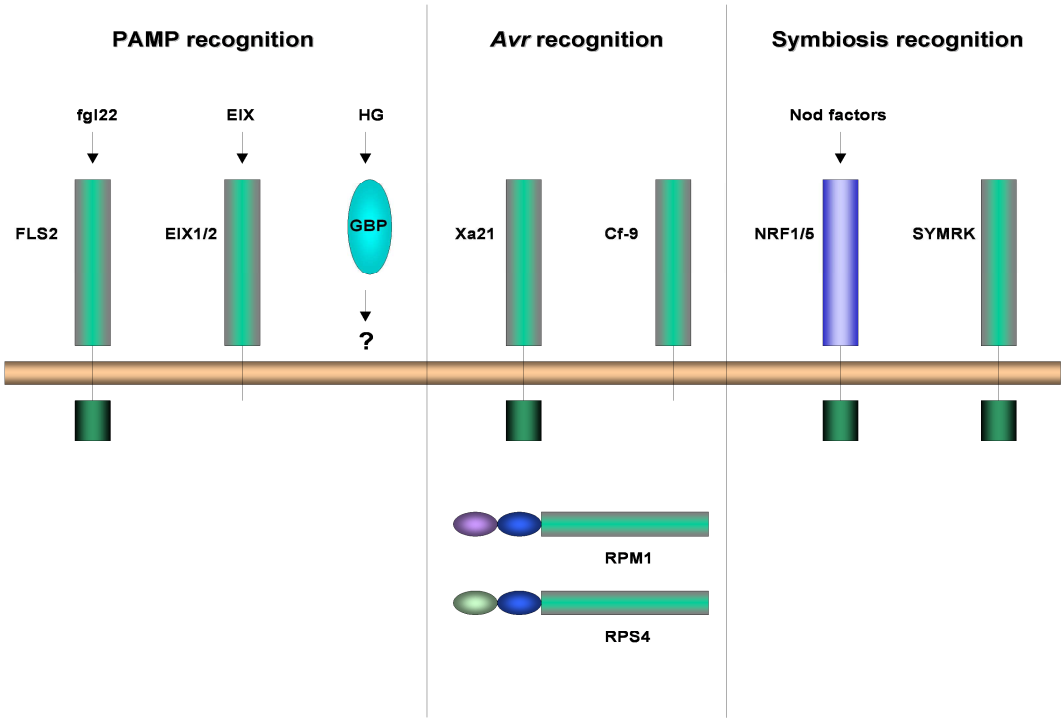


Fig. 7: Modular structure of recognition proteins in different interactions between plants and microorganisms (according to Zipfel & Felix, 2005).

1.3.2. Systemic acquired resistance

The systemic acquired resistance was initially examined and described by Chester in 1933 in the system of tobacco and TMV (Tabacco Mosaic Virus). After a local infection of tobacco with TMV, it was demonstrated that not only the local infection area led to increased resistance to a reinfection, but also healthy parts of plants showed a stronger immune response, which was referred to as the systemic acquired resistance (Ross, 1961b). Once this mechanism is active, it provides protection against various numbers of pathogens and can be maintained for up to several days and even weeks (Dangl *et al.*, 1996).

The systemic acquired resistance begins with the production of salicylic acid or its methyl ester (Taiz & Zeiger, 2000), which develops a few days after infection (Ryals *et al.*, 1996). This leads to the synthesis of pathogenesis-related proteins (PRPs) (Hammond-Kosack & Jones, 1997).

These proteins include glucanases and chitinases. As chitin and glucans are

major components of fungal cell wall and chitin is not present in higher plants, these proteins have been ascribed a role in the defense against fungal pathogens (Unger, 2001).

1.3.3. What is known about host and pathogen interaction in the *Vitis / Plasmopara* pathosystem?

The interaction between *Vitis* and *Plasmopara viticola* follows three distinct modes that partially correlate with differences in guard-cell morphology (Jürges *et al.*, 2009). (i) The American and Siberian *Vitis* species exhibit a true resistance, where the pathogen is arrested in its development early after attachment of the spores to the guard-cells. (ii) The Non-Siberian Asiatic species and the European wild *V. sylvestris* (or *V. vinifera* ssp. *sylvestris*) show moderate tolerance. In this interaction, *P. viticola* does not attach to the guard-cells but instead forms an abortive mycelium on the leaf surface. (iii) Susceptibility, where the plants are successfully infected, is the common case in cultivated grapevines. True resistance correlates with the presence of an additional cuticular rim at the inner side of the guard-cells (Jürges *et al.*, 2009).

Previous work comparing early pathogen development in a host-free (Riemann *et al.*, 2002) and a host-based system (Kiefer *et al.*, 2002), had identified several signals relevant for the successful infection of the host: (i) The release of zoospores from mature sporangia is accelerated by a signal released from the host. (ii) The morphogenesis of the germ tube is coordinated by spatial cues from the host which guides the organization of the cytoskeleton in the spore (Riemann *et al.*, 2002). (iii) The zoospores are targeted to the stomata by tracking factors that are released from the open stomata. Although the molecular nature of these signals was not known at the beginning of this thesis, it is clear that they are prime targets for the co-evolution in the interaction between *P. viticola* and its host. The tolerance of the Non-Siberian Asiatic *Vitis* species, for instance, is accompanied by a pronounced failure of zoospores to locate the guard cells, indicating that here the tracking factors have been lost.

It has been shown that the rate of synthesis of various phytolalexins (e.g. resveratrol) after infection with different pathogens is increasing for Vitaceae plants (Denzer, 1991). An increased tannin content, which acts on inactivating hydrolases of fungi, was observed in resistant American vines, and could not be detected in the susceptible European species (Bachmann & Blaich, 1979). In comparison, the American species *V. rupestris* responds to *P. viticola* infection with a hypersensitive reaction, whereas *V. vinifera displays* "only" a systemic response to infection. Busam *et al.* (1997) found the mRNA of the pathogen responsible for chitinase 3 in *V. rupestris* extracts only from the infection site, while in *V. vinifera*, the chitinase mRNA was found also in the subsequent three leaves indicating SAR.

1.4. Scope of the study

Based on the narrow host spectrum which is typical for oomycetes, it is likely that the evolution of the genus *Vitis* was also accompanied by the evolution of the pathogen. Investigations on *Plasmopara* in wild *Vitis* populations (*V. sylvestris*, as well as the American aboriginal populations) are completely lacking! This leads to exciting questions:

1. The species *P. viticola* is defined very vaguely. The host genus *Vitis* has experienced several stages of geographic isolation. Was this accompanied by a geographic isolation of the parasite? Could it be that several races or subspecies are “hidden” within the aboriginal populations? What species that are related to *Plasmopara viticola* coexist e.g. in Asia with wild species of *Vitis*, and are they able to infect these potential host species as effectively as *Plasmopara viticola* does infect cultured grapes?

2. The *Plasmopara / Vitis* pathosystem provides the unique case that we have a host population which was free of parasite due to geographic isolation, and that we have a historically recorded immigration of the parasite in this population. Does this lead to a radiation of this immigrating parasite? If so, how influenced this “host-jumping” of the parasite's phylogenetic relationship between

European and American *P. viticola* strains? What about its ancestor *Vitis sylvestris*? Is there a preadaptation to the newly introduced pathogen?
The objectives of this study are:

1. Genotypic identification – derived from DNA markers – of *P. viticola* strains from different areas all over the world. Isolates from the aboriginal populations in North America, from different regions of Europe are the focus of interest [reports on elusive species related to *P. viticola* in East Asia, such as *P. amurensis* and *P. cissii* (Grünzel 1959, Dick 2002) are doubtful]. Isolates from infected hosts are of special value.

2. Comparative analysis of host / pathogen interaction of different *Plasmopara* strains on different *Vitis* hosts (especially the European *Vitis* species) with focus on cell biological and molecular aspects. Which steps of the interaction (e.g. zoospore release, stomatal tracking, and mycelium development) are altered?

3. Since the European Wild Grape *Vitis sylvestris* represents a “naïve host”, it have to be tested, whether it harbors potential resistance factors as a preadaptation. These factors are expected to differ from those used in resistance breeding with North American resistant vines. As a prerequisite to utilize the potential of *Vitis sylvestris* as a resource, the genetic relationships between autochthonous European genotypes and potential gene flow from vineyards has to be elucidated using microsatellite analysis.

2. Materials and Methods

2.1. General Materials

2.1.1. Chemicals

Acridine Orange	Roth, Karlsruhe
Agar	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
β-Mercaptoethanol	Roth, Karlsruhe
Calcium hypochlorite	Roth, Karlsruhe
Cetyl trimethyl ammonium bromide (CTAB)	Roth, Karlsruhe
Chloroform, pa	Roth, Karlsruhe
n-Decanal	Roth, Karlsruhe
DNA marker	New England Biolabs, Frankfurt
Entellan	Merck, Darmstadt
Ethanol, pa	Roth, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe
Ethidium bromide	Roth, Karlsruhe
Hoechst 33258	Sigma-Aldrich, Taufkirchen
Isoamylalcohol (IAA)	Roth, Karlsruhe
Isopropanol (2-Propanol)	Roth, Karlsruhe
Lithium chloride	Roth, Karlsruhe
Loading buffer for DNA	New England Biolabs, Frankfurt
LR White	Resin, London
n-Nonanal	Roth, Karlsruhe
Paraformaldehyde	Roth, Karlsruhe
PIPES	Roth, Karlsruhe
Roti-Phenol	Roth, Karlsruhe
SYBR SAFE	Invitrogen, Darmstadt
Tris-(hydroxymethyl)-aminomethane	Roth, Karlsruhe

2.1.2. Primers for molecular phylogeny of *Vitis* species

Sequences of the chloroplast DNA today serve as the main data source to infer the phylogeny of plants. The chloroplast-genome holds some advantages over the nuclear DNA. First, it is small and the DNA is present in sufficient quantity. Second, the plastid genome has been extensively studied and therefore provides basic information for conducting comparative evolutionary studies. Third, the rate of nucleotide substitutions is relatively small, which allows the investigation of long-ago evolutionary processes (Clegg *et al.*, 1994).

Some studies on the molecular evolution were performed on intron and spacer sequences of the chloroplast DNA. One of the pioneering works was that of Chase *et al.*, 1993, in which the *rbcL* (the large subunit of RubisCO) sequence was used for phylogenetic studies in angiosperms. Further work followed with other genes, such as *ndhF* (NADH subunit 4L Dehydrodenase) (Olmstead & Sweere, 1994) and *matK* (Maturase K) (Johnson & Soltis, 1994). At the same time non-coding DNA-sequences of the chloroplast genome were examined. It was assumed that these sections are prone to variation and therefore would be suitable for studying low taxonomic levels. Taberlet *et al.* (1991) explored the use of the *trnT-trnL* / *trnL-trnF* regions (Fig. 8) and Johnson and Soltis (1994) the introns of the *trnK* / *matK* region (Fig. 9). Today, these two DNA sequences are among the commonly used sequences (77% of all phylogenetic studies) for the research of molecular evolution and the construction of phylogenetic trees in plants (Shaw *et al.*, 2005). Therefore primer pairs from the *trnL* / *trnF* and the *matK* region were selected in this study. The primers were synthesized by Sigma-Aldrich (Hamburg, Germany). For the molecular phylogeny of the *Vitis* species, following DNA regions were chosen: The non-coding regions of ctDNA *matK* / *trnK* and *trnL* / *trnF*.

The primer pair C (forward) and D (reverse) was used to amplify the intron of the *trnL* gene. Primer E (forward) and F (reverse) were used for amplification of the inter-genetic spacer between the *trnL* (UAA) and *trnF* 3' exon (GAA). A PCR with the primers *matK1* and *matK8F* amplified the DNA sequence between the 3' end of *trnK* 3' intron and the *matK* gene. *MatK5* and *matK6* were used to amplify the DNA segment between the 5' end of the *matK* gene and the 5' intron the *trnK* gene.

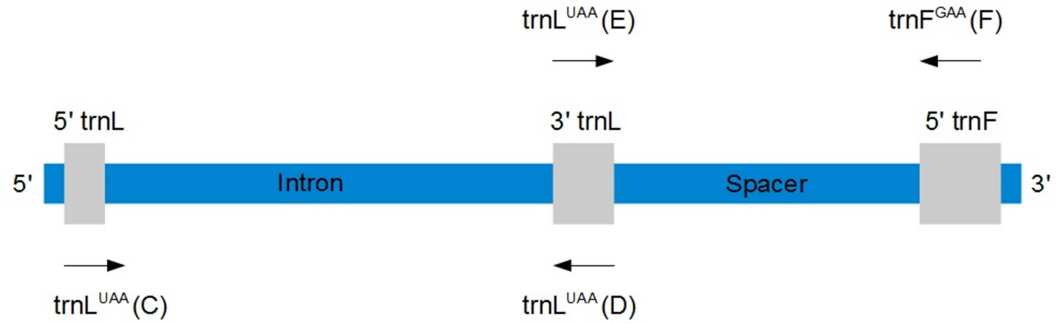


Fig. 8: Diagram of the *trnL/F* region.

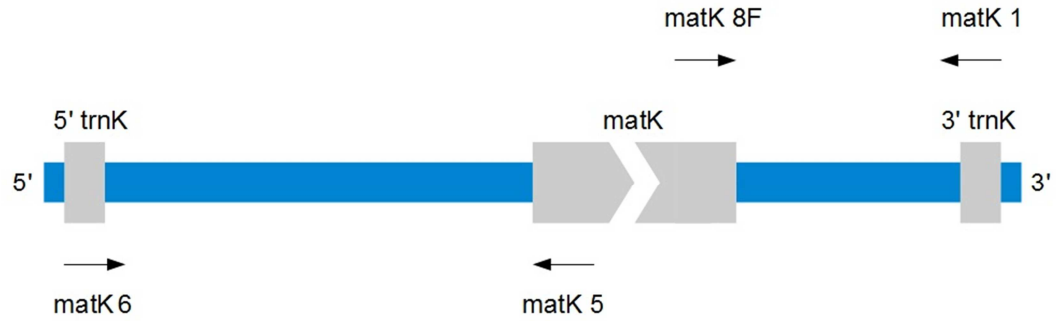


Fig. 9: Diagram of the *trnK* region.

Name	Sequence	Source
5´*trnL*^{UAA}F (C)	CGAAATCGGTAGACGCTACG	Taberlet *et al.* (1991)
3´*trnL*^{UAA}R (D)	GGGGATAGAGGGACTTGAAC	Taberlet *et al.* (1991)
3´*trnL*^{UAA}F (E)	GGTTCAAGTCCCTCTATCCC	Taberlet *et al.* (1991)
5´*trnF*^{GAA} (F)	ATTTGAACTGGTGACACGAG	Taberlet *et al.* (1991)
*matK*1	AACTAGTCGGATGGAGTAG	Shaw *et al.* (2005)
*matK*8F	TCGACTTTCTTGTGCTAGAACTTT	Shaw *et al.* (2005)
*matK*5	TGTCATAACCTGCATTTTCC	Shaw *et al.* (2005)
*matK*6	TGGGTTGCTAACTCAATGG	Shaw *et al.* (2005)

Tab. 3: Names, sequences and source of primers used for molecular phylogeny on *Vitis* species.

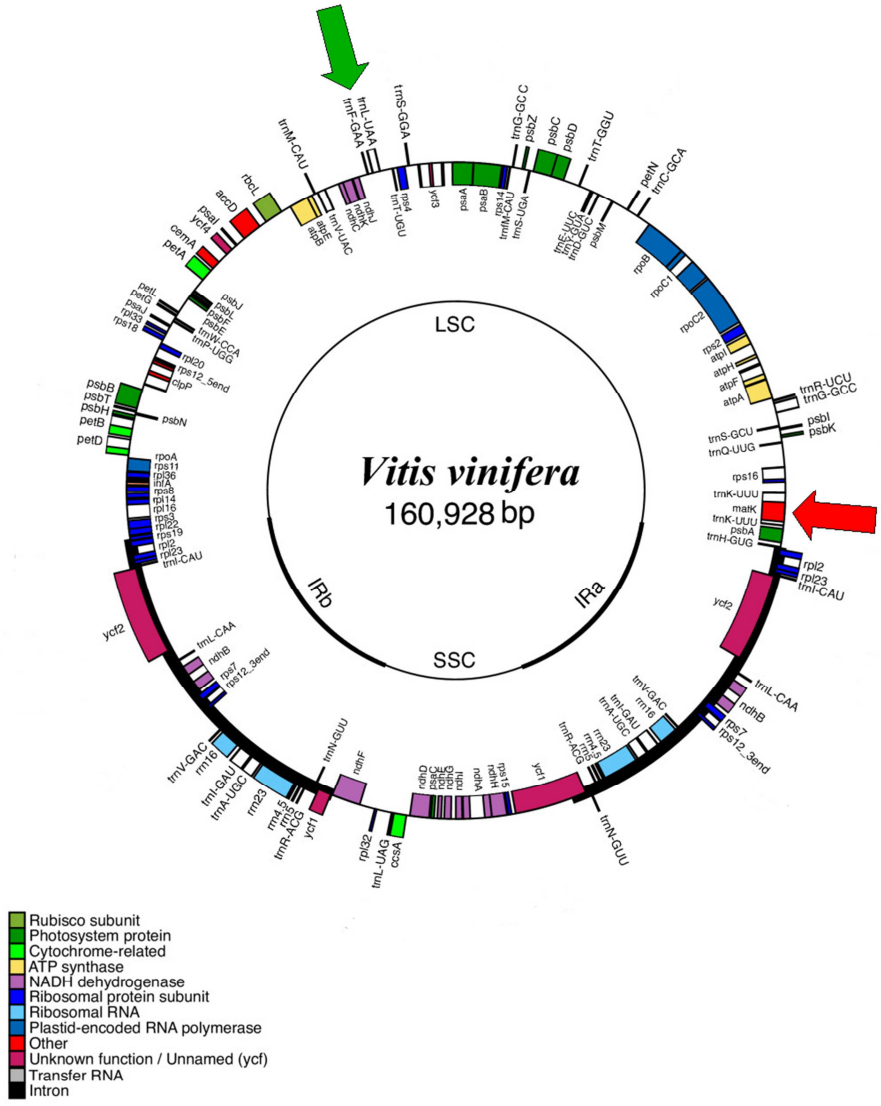


Fig. 10: Map of the chloroplast genome of *Vitis vinifera*. Green arrow: *trnL* / *trnF* region, red arrow: *matK* region (picture taken from Jansen *et al.*, 2006, modified).

2.1.3. Primers for microsatellite analysis of *Vitis sylvestris*

For the characterization of *Vitis sylvestris* plants, eight SSR (simple sequence repeats) were chosen (see below). Compared to other methods like RFLP (restriction fragment length polymorphism) analysis or RAPD (random amplified polymorphic DNA), SSR markers provide a quick and convenient way to characterize and compare closely related plants (Bowers *et al.*, 1996).

Additionally, SSR motives are common in almost all eukaryotic genomes and several SSR markers have been developed for many crop and non-crop plants (Sefc *et al.*, 1999). For this study, the grapevine SSR markers were used that have been defined by an international consortium as feasible to discriminate genotypes at the cultivar level and that were distributed over the different chromosomes. The primers were synthesized by Sigma-Aldrich. Forward primers (f) are fluorescently labeled (Tab. 4)

Name	Sequence	Source
VVMD7(f)	Cy3 – AGAGTTGCGGAGAACAGGAT	Bowers *et al.* (1996)
VVMD7(r)	CGAACCTTCACACGCTTGAT	Bowers *et al.* (1996)
VVMD25(f)	Fam – TTCCGTTAAAGCAAAAGAAAAAGG	Bowers *et al.* (1999)
VVMD25(r)	TTGGATTTGAAATTTATTGAGGGG	Bowers *et al.* (1999)
VVMD27(f)	Fam – GTACCAGATCTGAATACATCCGTAAGT	Bowers *et al.* (1999)
VVMD27(r)	ACGGGTATAGAGCAAACGGTGT	Bowers *et al.* (1999)
VVMD28(f)	Hex – AACAATTCAATGAAAAGAGAGAGAGAGA	Bowers *et al.* (1999)
VVMD28(r)	TCATCAATTTCGTATCTCTATTTGCTG	Bowers *et al.* (1999)
VVMD32(f)	Cy3 – TATGATTTTTTAGGGGGGTGAGG	Bowers *et al.* (1999)
VVMD32(r)	GGAAAGATGGGATGACTCGC	Bowers *et al.* (1999)
VVS2(f)	Fam – CAGCCCGTAAATGTATCCATC	Thomas & Scott (1993)
VVS2(r)	AAATTCAAAATTCTTATTCAACTGG	Thomas & Scott (1993)
VrZag62(f)	Hex – GGTGAAATGGGCACCGAACACACGC	Sefc *et al.* (1999)
VrZag62(r)	CCATGTCTCTCCTCAGCTTCTCAGC	Sefc *et al.* (1999)
VrZag79(f)	Hex – AGATTGTGGAGGAGGGAACAAACCG	Sefc *et al.* (1999)
VrZag79(r)	TGCCCCCATTTTCAAACTCCCTTCC	Sefc *et al.* (1999)

Tab. 4: Names, sequences and source of primers used for SSR analysis on *Vitis silsvestris*.

2.1.4. Primers for molecular phylogeny of *Plasmopara*

In addition to the use of chloroplast markers for phylogenetic analysis, mitochondrial markers are also commonly used such as *ypt1* (= *yp2*, yeast protein 2) or *cox2* (cytochrome c oxidase 2). In addition, nuclear markers as *ITS* (internal transcribed spacer), *IGS* (intergenic spacer) or *SSU* and *LSU* (small and large subunit rDNA) markers are widespread. It was shown that these markers can be utilized as a fast-running "molecular clock" for the phylogenetic classification of closely related organisms. Therefore, in order to discriminate different *Plasmopara viticola* isolates, *LSU* markers LR0R and LR6-O were chosen.

The primers were synthesized by IDT Integrated DNA Technologies, Inc. (Coralville, IA, USA).

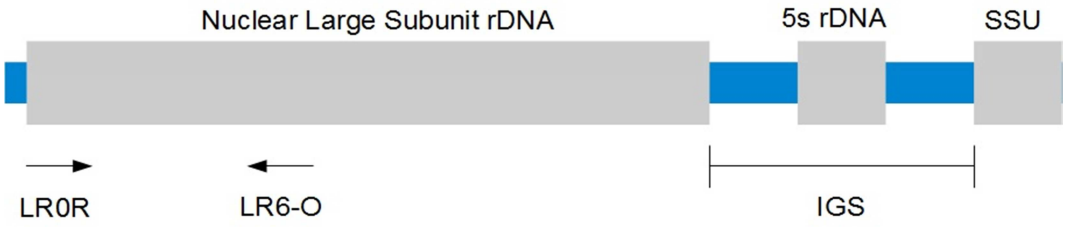


Fig. 11: Diagram of *LSU* rDNA.

Name	Sequence	Source
LR0R(f)	GTACCCGCTGAACTTAAGC	Moncalvo *et al.* (1995)
LR6-O(r)	CGCCAGACGAGCTTACC	Riethmüller *et al.* (2002)

Tab. 5: Names, sequences and source of primers used for molecular phylogeny on *Plasmopara viticola*.

2.1.5. Plant material for molecular phylogeny of *Vitis*

All plant samples were obtained from New York State Agricultural Experiment Station, Cornell University NY, USA. They were cultivated in a greenhouse with additional light from 7.00 am – 10.00 pm. The following *Vitis* genotypes were used for the experiment:

Vitis amurensis
Vitis riparia
Vitis vinifera cv. “Riesling”
Vitis hybrid “BR 14” (*V. rupestris* 66-4 x S 14664)
Vitis hybrid “Horizon” (Seyval x Schuyler)
Vitis hybrid “Wilcox 321”(Blue Jay x MN 242)

2.1.6. Plant material for microsatellite analysis of *Vitis sylvestris*

The following wild plants were collected and their GPS location was recorded:

42 *Vitis sylvestris* plants were collected from the peninsula “Ketsch” in the Rhine-river, southern Germany.
This collection was complemented by:
1 *Vitis sylvestris* plant originated from the Botanical Garden of Karlsruhe University, Germany.
2 *Vitis sylvestris* were collected in Hördt, southern Germany.
5 *Vitis sylvestris* originated from Danube valley, Austria.

For comparison, *Vitis vinifera* cv. “Müller Thurgau”, *Vitis rupestris*, *Vitis riparia* and *Vitis amurensis* were used as references. They also originate from the Botanical Garden of Karlsruhe University. The SSR marker lengths for seven cultured grapes: “Cabernet”, “Riesling”, “Chardonnay”, “Pinot noir” and “Pinot blanc”, “Traminer” and “Silvaner”, were obtained mainly from “*Organisation Internationale de la Vigne et du Vin”*, 2nd edition of the OIV descriptor list for grape varieties and *Vitis* species database (OIV, 2001).

2.1.7. Pathogenic material (*Plasmopara viticola* isolates)

Plasmopara viticola isolates were collected in the USA and Germany, or obtained from the WBI (Weinbau Institut, Freiburg, Germany), or Syngenta AG, (Basel, Switzerland) (Tab. 6). GPS data of each collection site were recorded. Isolates were propagated, if necessary, on detached leaves, which were disinfected by washing in a Calcium hypochlorite solution (430 mg/500 ml dH_2O) for 20 seconds and then in water. Then the infected leaf was brought in contact with the acceptor leaf to transmit the pathogen.

Pathogen	Host	Isolate	Year	Location
Plasmopara viticola	*Vitis vinifera*	WBI 1	2009	Germany, Freiburg (Baden-Württemberg)
Plasmopara viticola	*Vitis vinifera*	WBI 2	2009	Germany, Freiburg (Baden-Wuerttemberg)
Plasmopara viticola	*Vitis vinifera*	GI	2007	Germany, Gundelsheim (Baden-Württemberg)
Plasmopara viticola	*Vitis vinifera*	SI	2007	Germany, Sasbach (Baden-Württemberg)
Plasmopara viticola	*Vitis vinifera*	II	2007	Germany, Ihringen (Baden-Württemberg)
Plasmopara viticola	*Vitis vinifera*	WI	2007	Germany, Weinsberg (Baden-Württemberg)
Plasmopara viticola	*Vitis vinifera*	Syngenta	2009	Switzerland, Basel (Basel-Stadt)
Plasmopara viticola	*Vitis vinifera*	PvI	2006	USA, Geneva, NY
Plasmopara viticola	*Vitis vinifera*	JI	2006	USA, Seneca County, NY
Plasmopara viticola s.l.	*Vitis riparia*	MI	2006	USA, Marathon, NY
Plasmopara viticola	*Vitis vinifera*	KI	2006	USA, Keedysville, MD
Plasmopara viticola	*Vitis vinifera*	TxI	2007	USA, Cat Spring, Tx
Plasmopara viticola	*Vitis vinifera*	TxII	2007	USA, Cypress, Tx
Plasmopara viticola s.l.	*Vitis vinifera*	TxIII	2007	USA, Hill County, Tx
Plasmopara viticola s.l.	*Vitis vinifera*	TxIV	2007	USA, Hill County, Tx

Tab. 6: *Plasmopara viticola* isolates were collected in the USA and Germany or obtained from the WBI or Syngenta.

2.2. Methods

2.2.1. Isolation of genomic DNA from plants

DNA was extracted from leaf tissue by using either the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer or the CTAB (cetyl trimethyl ammonium bromide) method (Doyle & Doyle, 1987).

Step one: In both cases, 50 mg fresh plant material was cooled in liquid nitrogen and ground to a fine powder. For the CTAB method, 700 µl of warm CTAB buffer (67° C, 3% w/v CTAB, 1.4 M NaCl, 0.3 M Tris-HCl (pH 8), 25 mM EDTA) and 1.75 µl proteinase K (Sigma-Aldrich, Taufkirchen, Germany) were added to the homogenate and incubated for 10 minutes at minutes at room temperature on the overhead tumbler. It was centrifuged for 5 minutes at 5000 rpm and the upper phase transferred into a fresh reaction vessel. In step three, 700 µl of chloroform / isoamyl alcohol (24:1) was added and mixed for 10 minutes at room temperature on the overhead tumbler and centrifuged for 10 minutes at 5000 rpm. In step four, the upper phase was transferred to a fresh 1.5 ml reaction tube. After adding of 0.6 volumes of ice cold (–20° C) pure isopropanol and 0.1 volumes LiCl (3 M) the solution was mixed by shaking and incubated for 30 minutes on ice and centrifuged for 10 minutes at 7500 rpm immediately thereafter. Finally, the pellet was washed with 70% ethanol and dried for 10 minutes, before the DNA was dissolved in 200 µl distilled water.

2.2.2. Isolation of genomic DNA from *Plasmopara viticola*

Sporangia and conidia were carefully collected from the leaf surface by using a Pasteur pipet. The DNA from the collected material was extracted using the MOBIO Laboratories UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer's protocol.

2.2.3. DNA quantification

The amount of extracted DNA was determined by photometry at wavelengths of 250 nm, 260 nm and 280 nm. The UV spectrum of DNA shows a maximum at 260 nm in aqueous solution. As known, pure DNA, free of protein, has a ratio of extinction at different wavelengths (1.85 at E260 / E280 and 2.2 at E260 / E250). Pollutions by proteins become apparent in a shoulder at 280 nm. Therefore, the second value was taken at 280 nm to detect pollutions.

2.2.4. PCR amplification

For DNA amplification of both *Vitis* and *Plasmopara* isolates, about 25 ng of isolated genomic DNA were used as template.

The PCR mixture contained:
2 µl Ex Taq buffer (10x), (Takara Bio Inc., Otsu, Japan)
2 µl Ex dNTPs, (Takara Bio Inc., Otsu, Japan)
12 pmol primer, each
Template
0.5 µl (1.5 Units) Ex Taq DNA polymerase, (Takara Bio Inc., Otsu, Japan)
A total volume of 20 µl was made up with H_2O.

The following PCR program was used:
Cycle 1) 95 ° C for 5 minutes
Cycle 2) 95 ° C for 1 minute
Cycle 3) 60 ° C for *Vitis* primer, 63 ° C for Downy Mildew primer, for 1 minute
Cycle 4) 72 ° C for 2 minutes
Cycles 2) - 4) were repeated 36 times.
Cycle 5) 72 ° C for 4 minutes, then 4 ° C ad infinitum

2.2.5. Separation of PCR fragments

For analysis of PCR fragment size, as well as separation and purification, Agarose gel-electrophoresis was used [the gels contained: 1% Agarose in TAE buffer (Tris-acetate-EDTA, pH 8.3) and 0.03% SYBR SAFE]. The PCR fragments were cut from the gel with a razor blade under blue light and purified using Macherey & Nagel Nucleo Spin Extract kit (Macherey & Nagel, Düren, Germany) according to manufacturer's instructions.

2.2.6. Sequence analysis of *Vitis* species

The DNA was sequenced by GATC, Konstanz. Sequence comparisons were made using the BLAST search engine from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST), (Levenstein, 1966). Sequences were aligned with ClustalX (1.81) (Thompson *et al.*, 1997). Alignment errors were edited manually using the "Seaview" program for Windows. In case of *Plasmopara* alignments the mafft software (Katoh *et al.*, 2002), version 6, using the Q-INS-i algorithm (Katoh & Toh, 2008) was used without manual editing (Schröder *et al.*, submitted).

2.2.7. Construction of phylogenetic trees

Phylogenetic trees were constructed with MEGA 4.0 (Tamura *et al.*, 2007) for Maximum Parsimony and Minimum Evolution inference, with all parameters set to default values, and with RAxML (Stamatakis, 2006) in the webserver version (Stamatakis *et al.*, 2008) for Maximum Likelihood inference, estimating the proportion of invariable sites and with all parameters set to default values. In both cases, 500 bootstrap replicates were carried out for testing tree robustness.

2.2.8. PCR amplification for SSR-analysis

For DNA amplification about 25 ng of isolated genomic DNA were used as template.

The PCR mixture contained:

2 µl r Taq buffer (10x), (Takara Bio Inc., Otsu, Japan)

2 µl r dNTPs, (Takara Bio Inc., Otsu, Japan)

12 pmol primer, each

Template

0.5 µl (1.5 Units) r Taq DNA polymerase (Takara Bio Inc., Otsu, Japan)

A total volume of 20 µl was made up with H_2O.

The following PCR program was used:

Cycle 1) 95 ° C for 5 minutes

Cycle 2) 95 ° C for 15 seconds

Cycle 3) 53 ° C for VVMD7, VVMD32 and VrZag79 for 30 seconds,
58 ° C for VVS2, VVMD27 and VrZag62 for 30 seconds and
60 ° C for VVMD25 and VVMD28 for 30 seconds

Cycle 4) 72 ° C for 30 seconds

Cycles 2) - 4) were repeated 36 times.

Cycle 5) 72 ° C for 4 minutes, then 4 ° C ad infinitum

2.2.9. Microsatellite analysis and construction of *Vitis sylvestris* dendrograms

Mircrosatellite analysis was conducted by the company GATC (Konstanz, Germany). Fragment lengths of the random repeats were evaluated by GeneMarker and analyzed with Identity 1.0 (Wagner & Sefc, 1999). A Microsat output file was created, which was then used to calculate a distance matrix by Microsat2, using the option “chord distance”. The distance matrix was used in MEGA 4.0 (Tamura *et al.*, 2007) to reconstruct a Neighbour Joining Tree with default settings.

2.2.10. Distance matrix of *Vitis silvetris* plants collected at "Ketsch"

GPS data of the *Vitis sylvestris* plants collected at "Ketsch" were inserted into Google earth and distances were measured creating a matrix. The distance matrix was used in MEGA 4.0 (Tamura *et al*., 2007) to reconstruct a Neighbour Joining Tree with all settings on default.

2.2.11. Stomata morphology

Stomata morphology was analyzed in *Vitis* hybrids "BR 14", "Horizon" and "Wilcox 321" and in *Vitis sylvestris* species from "Ketsch" (K) and "Hördt" (H) (H13, K15, K20, K39, K53, K83, K94, K95, K99 and K103), in cultured grapes *Vitis vinifera* cv. "Müller Thurgau" and *Vitis vinifera* cv. "Riesling" and in wild American *Vitis* species *Vitis riparia* and wild Asian species *Vitis amurensis*. In the first step, small specimens of leaf tissue were fixed in 4% Paraformaldehyde (prepared freshly in 3 mM EDTA, 50 mM PIPES, pH 7.0). The specimens were washed with 50 mM PIPES pH 6.0 three times for 30 minutes. Then they were dehydrated through an ethanol series (30%, 50%, 70%, 90%, 100% Ethanol, 30 minutes each). In the second step, the specimens were transferred into a 2:1 Ethanol / LR White Resin solution and incubated on an overhead tumbler over night. Immediately after, the specimens were transferred into a 1:1 Ethanol / LR White Resin solution for 4 hours, afterwards into a 1:2 Ethanol / LR White Resin solution again for 4 hours, and finally into a 100% LR White Resin solution and incubated on a tumbler over night. In the third step, the leaf pieces were embedded in gelatin capsules and baked at 50° C for 72 hours. The capsules were trimmed with a razor blade. Semi-thin sections of 5 µm width were cut with a conventional microtome, attached to chrom-gelatine, and dried for 5 hours. In the fourth step, sections were stained for 5 minutes with a 1% (w/v) aqueous solution of acridin orange, adjusted to pH 7. After washing with destilled water, the specimens were dried, covered with a drop of Entellan, and then covered for a day with a coverslip bearing a 5 g weight. Finally, the sections were analyzed under an epifluorescence microscope (Axioskop, Zeiss, Göttingen, Germany)

using the filter set 1 (excitation 365±12 nm, beam splitter 395 nm, emission through a long-pass filter 397 nm)

2.2.12. Chemotaxis assay

Freshly collected sporangia were adjusted to $8 \cdot 10^4$ sporangia/ml with distilled water and prehatched for 2 hours at 25° C under continuous white light (5000 $Lx \cdot m^{-2}$). An aliquot of 50 µl was placed in the chamber of a hemacytometer (Fuchs-Rosenthal, Thoma, Freiburg, Germany), and digital images of all individual sampling squares recorded under differential interference contrast (Axioskop and Axiovision, 4.5 or newer, Zeiss, Göttingen, Germany) using a 20x Neofluar objective (Zeiss, Göttingen, Germany). At time 0 minutes either a small (1 mm x 1 mm) sample of a fresh *Vitis* leaf or, alternatively, 0.1 µl of the test substance were placed close to the chamber such that there was no direct contact with the suspension, but only through the gas phase. After 15 minutes, the sampling squares were recorded again and relative frequencies in each square were scored. As test substances, either distilled water, cis-hexenyl acetate, n-nonanal, or n-decanal were used. Trajectories of individual zoospores were recorded at intervals of 1 second and the xy-position determined from the images using the Axiovision digital recording software (Zeiss, Göttingen, Germany).

2.2.13. Effect of nonanal and decanal on infection

"Müller-Thurgau" leaves from the 4th to the 6th position from the shoot tip were harvested. The samples were disinfected by washing in calcium hypochlorite (430 mg/500 ml dH_2O) for 20 seconds and then rinsed with water. Following this procedure, the leaves were air-dried on kitchen tissues.

Eight leaf discs of 12 mm diameter were infected with two different *Plasmopara viticola* isolates (WBI 2 and Syngenta) and different concentrations of nonanal, decanal and the solvent (1 µl n-nonanal, 0.1 µl n-nonanal, 0.01 µl n-nonanal, 0.01 µl n-decanal, 1 µl Ethanol and 1 µl H_2O). The leaf discs were inoculated for

30 minutes with a sporangia suspension (4000 sporangia/µl). After 30 minutes, the leaf discs were transferred onto water agar (0.8%) in a cross formation and a small, specified, volume of either n-nonanal or n-decanal and control solution was placed on a small patch of filter paper (10 mm x 10 mm) in the center of the petridish and sealed with Parafilm.
The leaf discs were incubated at 25° C with continuous white light (5000 $Lx \cdot m^{-2}$) for additional 6 days, before the incidence of infection was scored from digital images recorded with an Olympus SZ-CTV binocular microscope, equipped with an Olympus Camedia C-5060 wide zoom camera using Assess for Windows 1.0 software measuring the area covered by sporulating *P. viticola* and necrotic lesions. For each data point, three independent series using different inocula were conducted with eight individual leaf discs.

2.2.14. Cross Infection

For cross infection experiments, six different grapevine species were used: *Vitis vinifera* cv. "Riesling", *Vitis amurensis*, *Vitis riparia* and the hybrids "BR 14", "Horizon" and "Wilcox 321".
Leaves from the 4th to the 6th position from the shoot tip were harvested. They were desinfected by washing in calcium hypochlorite (430 mg/500 ml dH_2O) for 20 seconds. Then the leaves were washed with water and rinsed with several fungicides, which are known to be ineffective for *Plasmopara viticola*: Nova (Myclobutanil 40%, Dow AgroScience, Munich), 0.5 g/l, Endura (Boscalid, BASF, Ludwigshafen), 0.3 g/l, and TOPSIN (Bayer, Monheim), 0.4 g/l. The leaves were then air-dried on kitchen tissues.
12 leaf discs of 12 mm diameter from each *Vitis* species were prepared for all 11 different *Plasmopara viticola* isolates and inoculated for 30 minutes with a sporangia suspension (4000 sporangia/µl). After 30 minutes, the leaf discs were transferred onto water agar (0.8%) in a petridish and sealed with Parafilm.

The leaf discs were incubated at 25° C with continuous white light (5000 $Lx \cdot m^{-2}$) for additional 8 days, when the incidence of infection was scored from digital images recorded with a Olympus SZ-CTV binocular and a Olympus Camedia C-

5060 wide zoom camera. The Assess for Windows 1.0 software was used to measure the area covered by sporulating *P. viticola* and necrotic lesions. For each data point and each of the European isolates (WBI 2 and Syngenta) three independent series using different inocula were conducted. Due to limitations in the material, for the American isolates, only one series could be conducted.

2.2.15. Fitness scores

From each *Vitis* species and each Downy Mildew isolate, infected leaf discs (obtained from the cross infection experiment) were transferred to a 70% Ethanol solution and shaken thoroughly. The solution was transferred to a 1.5 ml reaction tube and centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded and 1 ml water and 1 µl Hoechst 33258 solution (100 µg/ml) and was added to the sediment. The supernatant was analyzed under an epifluorescence microscope (Axioskop, Zeiss, Göttingen, Germany) using the filter set 1 (excitation 365 ± 12 nm, beam splitter 395 nm, emission through a long-pass filter 397 nm, Fig. 12). The average number of nuclei and the average mortality were scored from 100 sporangia per sample.

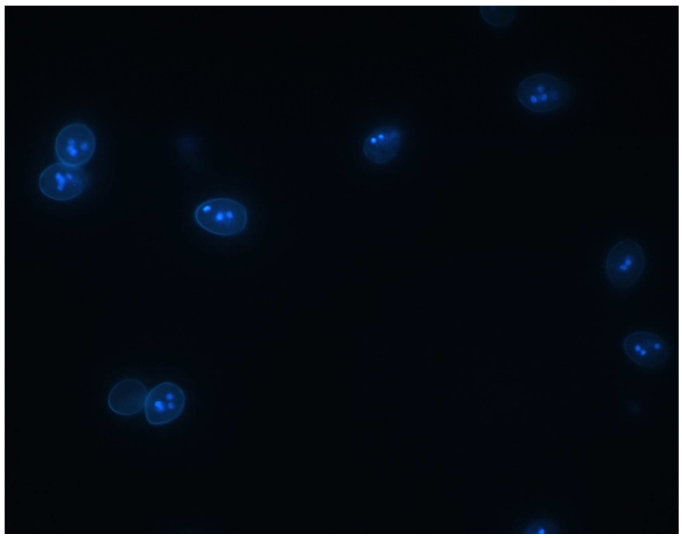


Fig. 12: Sporangia of *Plasmopara viticola* with fluorescent nuclei.

2.2.16. *Vitis sylvestris* infection assay

For the infection assay, detached leaves from 10 *Vitis sylvestris* species (H13, K15, K20, K39, K53, K83, K94, K95, K99 and K103) and three grapevine cultivars as a control (*Vitis vinifera* cv. “Müller-Thurgau”, *Vitis vinifera* cv. “Regent” and *Vitis vinifera* cv. “Riesing”) were used. The cultivar “Regent” is considered resistant to Downy Mildew. For treatment, the 4th to 6th leaf from the shoot tip was harvested and put upside down in a petridish on wet filter paper (3 ml water). Five leaves from five different plants for each grapevine species were tested. The leaves were sprayed five times with a mist of a *Plasmopara viticola*-suspension (50000 sporangia/ml). The petridish was sealed and placed in a dark room over night at 21° C. The next day, excess water was removed from each sample under sterile conditions and the petridish was resealed. Then, the samples were placed into a climate chamber for six days at 21° C with 12 hours (5000 $Lx \cdot m^{-2}$) regime. The percentage of the infected leaf surface was measured. The results were divided into seven classes expressing percentage of leaf surface infection (0%, < 5%, < 10%, < 25%, < 50%, < 75%, < 100%). The experiment was repeated three times.

3. Results

Plasmopara viticola, the false downy mildew, is an obligate biotrophic grapevine pathogen, originating from the American continent and was introduced to Europe in the 1860s.

In America, *P. viticola* underwent a co-evolution with wild American grapevine species, which are resistant to the pathogen. This might have led to specifications of some pathogen isolates according to the host grapevine species' physiology and therefore to phylogenetic changes in the pathogen.

The American host grapevine species on the other hand, in order to obtain resistance against the pathogen, underwent also physiological and morphological changes by co-evolving with *P. viticola*.

In contrast, in Europe, *P. viticola* met two naïve hosts: *Vitis vinifera*, which is highly susceptible to the pathogen, and its ancestor, the wild European grapevine species *Vitis sylvestris*, whose degree of resistance was never properly evaluated. This so called "host jump" might also have induced phylogenetic changes in the pathogen.

3.1. Genotypic identification and comparative analysis of host / pathogen interaction

The first part of this study focuses on pathogen phylogeny as well as host phylogeny and the specification of host and pathogen interaction.

To get insight into *P. viticola's* cryptic diversity, phylogenetic trees of the pathogen, based on genetic markers, were created. Additionally, the phylogenetic relationship of grapevine species, which were used for subsequent experiments, was determined.

Cross infection assays of six grapevine species and 11 *P. viticola* isolates were performed and infection intensity and necrotic lesions were measured in order to compare host specific reaction and fitness scores for each *P. viticola* isolate was measured.

Finally host specifications as nonanal emission and cuticular inner rims were investigated.

3.1.1. Phylogeny of Plasmopara viticola

For a better understanding of *P. viticola* and its interactions with its host *Vitis*, a more detailed phylogeny of the pathogen had to be carried out. Up to now it was still unknown, if there are “hidden” subspecies of *P. viticola*, which might have co-evolved differently with different host species and therefore developed different infection patterns.

Therefore, tree topology was carried out by several mitochondrial and nuclear markers: (i) The mitochondrial *cox2* according to the protocol of Hudspeth *et al.* (2000). (ii) For amplification of *ypt1* the primers Ypt1F described in Chen and Roxby (1996) and Ypt4R described in Moorman *et al.* (2002) were used. (iii) Partial *nrLSU* was amplified according to the protocol given in Riethmüller *et al.* (2002) and Moncalvo *et al.* (1995). The results were combined with data from M. Thines, Johann Wolfgang Goethe University, Department of Biological Sciences, Institute of Ecology, Evolution and Diversity, Siesmayerstr. 70, D-60323 Frankfurt (Main), Germany.

The tree topology for the *Plasmopara* strains infecting *Vitis* was found to show similar results in phylogenetic reconstructions based on following sequences*: ypt1* (mitochondrial, Fig. 13), partial *nrLSU* (Fig. 14) and *cox2* (mitochondrial, Fig. 15). No conflicts were observed in any of the analyses. All three genes revealed three distinct phylogenetic lineages within *Plasmopara* from *Vitis* in both Minimum Evolution and Maximum Likelihood inference. One of these clades (clade 1) contained samples from both North America and Europe, while the other two lineages (clades 2 and 3) contained only American isolates.

The analysis of *ypt1* offers the highest phylogenetic resolution of the genes investigated (Fig. 13). Clade 1 was divided into two subclades. First subclade, containing the European isolates, which were all identical in sequence, and the second containing the North American isolates, which showed genetic variation between the samples from Texas and those from North-East America (Fig. 13). Clade 1 contained the samples collected from *Vitis vinifera* and cultivated hybrids. Clade 2 contained samples from *Vitis vinifera* [TxIII was collected from the grapevine variety “Barbera”, TxIV was collected from grapevine variety “C1613” (*Vitis solonis* x *V. vinifera* var. “Othello 1613”)] and clade 3 contained samples from *Vitis riparia* (Fig. 13).

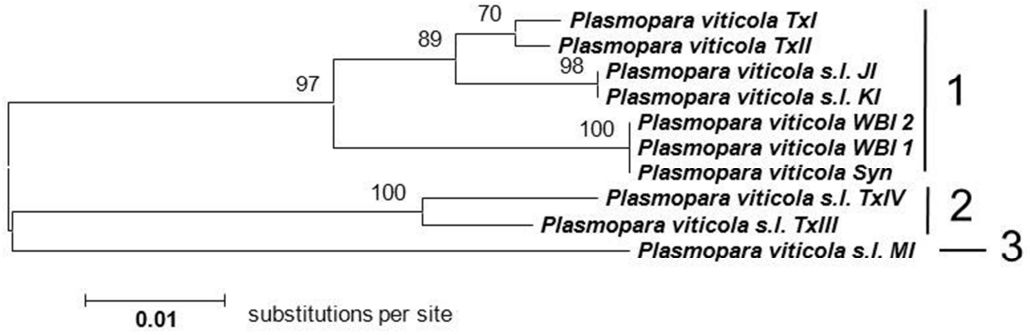


Fig. 13: Maximum evolution tree based on *ypt1*. Bootstrap analysis was set to 500 replicates.

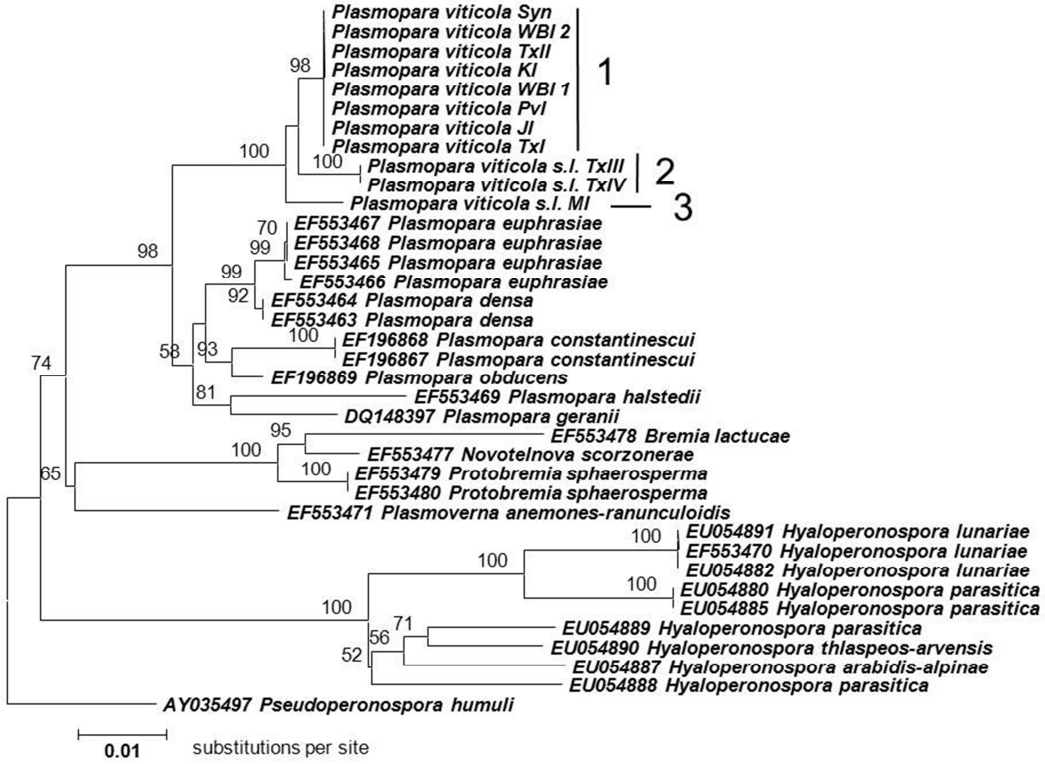


Fig. 14: Maximum evolution tree based on *nrLSU*. Bootstrap analysis was set to 500 replicates.

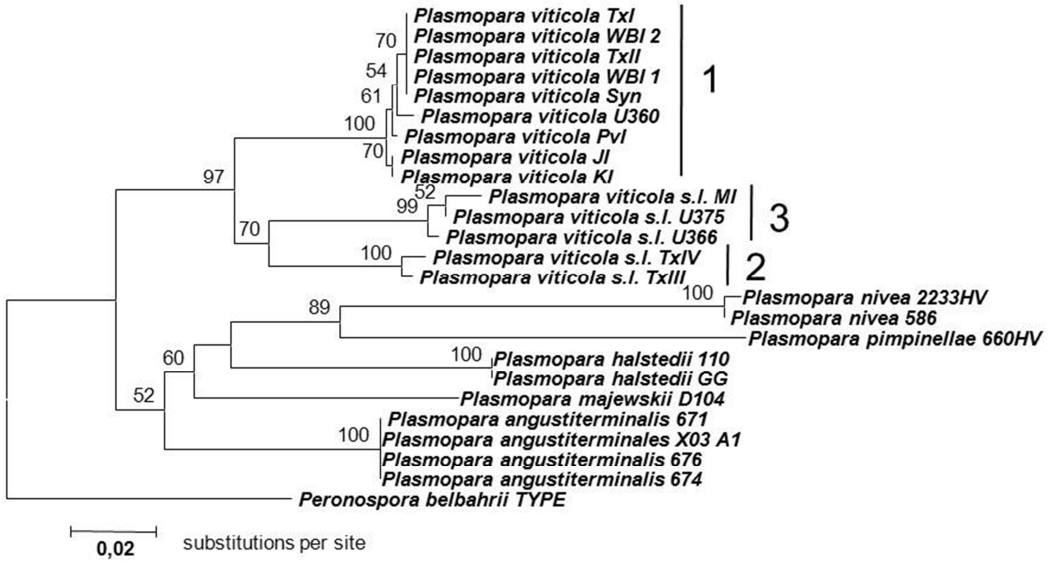


Fig. 15: Maximum evolution tree based on *cox2*. Bootstrap analysis was set to 500 replicates.

3.1.2. Phylogeny of *Vitis* species

In order to relate the phylogeny of *P. viticola* isolates with the *Vitis* species, which were used for cross infection experiments (see ahead), a phylogenetic tree of *Vitis* was created by using preexisting data (Tröndle *et al.*, 2010) as well as sequences of the grapevine species used for the actual cross infection experiments.

Six different grapevine species have been used for cross infections (*V. amurensis*, *V. riparia* and *Vitis vinifera* cv. “Riesling” and the hybrids “BR 14”, “Horizon” and “Wilcox 321”). The grapevine species were selected according to observed infection severity towards downy mildew (classified by staff members of Plant Genetic Resources Unit (PGRU), Cornell, NY, USA), and divided into “severity classes”.

Molecular phylogeny of the different *Vitis* species was reconstructed based on combined sequence analysis of plastid markers of *trnL* intron, inter-genetic spacer *trnL* (UAA) / *trnF* 3' (GAA) and *matK* regions *matK1* / *matK8F* and *matK5* / *matK6*. *Vitis* phylogenetic tree is presented in figure 16 as a condensed Maximum Parsimony tree, with a cut-off value of 50%, which combines all branches with a lower bootstrap value than 50. The *Vitis* species marked with an asterisk in figure 16, are the ones used for cross infection experiments.

The phylogenetic tree consists of three clades. Each clade represents the continental distribution of the *Vitis* species (Asian, European and American).

The condensed Maximum Parsimony tree showed *V. amurensis* (VAmu2 *) branching with *V. betulifolia* instead of *V. amurensis* species VAmu1. The cultivar “Riesling” clustered with the other European *V. vinifera* species and due to the low bootstrap values, most American *Vitis* species cluster in one branch. Therefore, despite of the difference of two base pairs between both *V. riparia* accessions, *V. riparia* (VRip2 *) takes place next to *V. riparia* (VRip1) in the condensed Maximum Parsimony tree. The hybrids “BR 14”, “Horizon” and “Wilcox 321” are also located in the American clade. *Rhoicissus rhombifolia* and *Ampelopsis* spec. were used as outgroups.

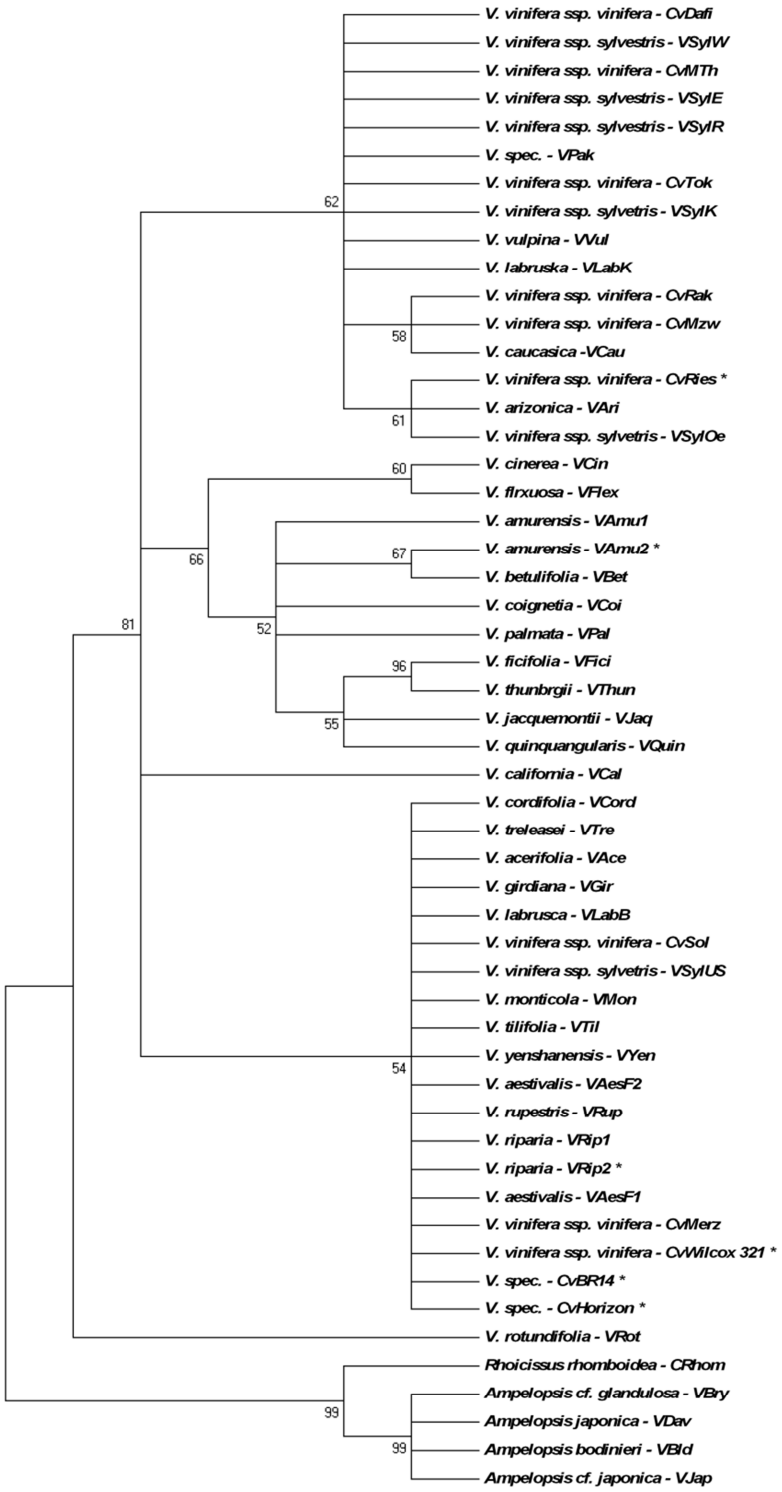


Fig. 16: Condensed maximum parsimony tree (cut-off value = 50%). Bootstrap analysis was set to 500 replicates.

3.1.3. Cross infection assay

To compare pathogen aggressiveness of different *P. viticola* isolates with host specific defense intensity cross infection experiments with several *Vitis* species (selected according to severity levels of downy mildew infection) were performed.

Leaf discs of six different grapevine species were inoculated with 4000 sporangia/µl of each *P. viticola* isolate. Infection rates (percentage of leaf surface infection and necrotic leaf surface) were measured over all *P. viticola* isolates and grapevines plants and are presented in figures 17 and 18.

For most isolates the cross infection experiments could be performed only one time. Only for the German and Swiss isolates “WBI2” and “Syngenta” three independent experiments could be performed.

In a cross infection assay, *V. amurensis* showed the highest infection values to all *P. viticola* isolates compared to all other grapevine species (average of infected leaf surface of 27.5% and approximately 40% necrotic leaf surface, data not shown). The cultivar “Riesling” showed an average of 16.4% infected leaf surface, measured over all tested isolates, and a necrotic leaf surface of 7.2%. The hybrid “Horizon” showed values of 13.4% and 3.7%, respectively. The grapevine hybrids “BR 14” and “Wilcox 321”, and the wild American grapevine species *V. riparia* showed lower rates (infected leaf surface < 4%; necrotic lesions < 2%, measured over all *P. viticola* isolates, data not shown). Differences in infection rates were visible among the grapevine species for each *P. viticola* isolate (Fig. 17 and Fig. 18).

V. amurensis showed no signs of infection for the isolates “WBI2” and “Syngenta”, and only small necrotic lesions (< 2% of leaf surface). For the isolate “TxII”, *V. amurensis* showed 12% infected leaf surface and 94% necrotic leaf surface. For the isolate “TxIV”, *V. amurensis* showed 58% infected leaf surface and 24 % necrotic leaf surface. All other isolates demonstrated average range of infection between 24-41%, and between 31-98% necrotic leaf surface (Fig. 17 and Fig. 18).

The hybrid “BR 14”, showed low infection rates to the isolates “WBI2” (23% infected leaf surface and 5% necrotic leaf surface) and “Syngenta” (9% infected leaf surface and 2% necrotic leaf surface).

The *Vitis* hybrid "Horizon" showed the highest infection rates (59% infected leaf surface) to *P. viticola* isolate "JI", followed by the isolate "TxIV" (27%) and "KI" (19%). All other isolates demonstrated infection < 6% infected leaf surface.

The cultivar "Riesling" showed infection rates ranging between 15-33% to most isolates, with exception of the isolates "KI", "KVI" and "TxII" (< 7% infected leaf surface, Fig 17).

V. riparia demonstrated low infection rates to all *P. viticola* isolates (< 3% infected leaf surface).

The hybrid "Wilcox 321" showed moderate infected leaf surface values to the isolates "JI" (25%) and "KI" (13%), and < 1% to all others isolates.

Both grapevine varieties, *V. riparia and* hybrid "Wilcox 321", showed only little necrotic lesions (< 5%, Fig 23). Test results of the *P. viticola* isolates "MI" and "TxIII" were not obtained.

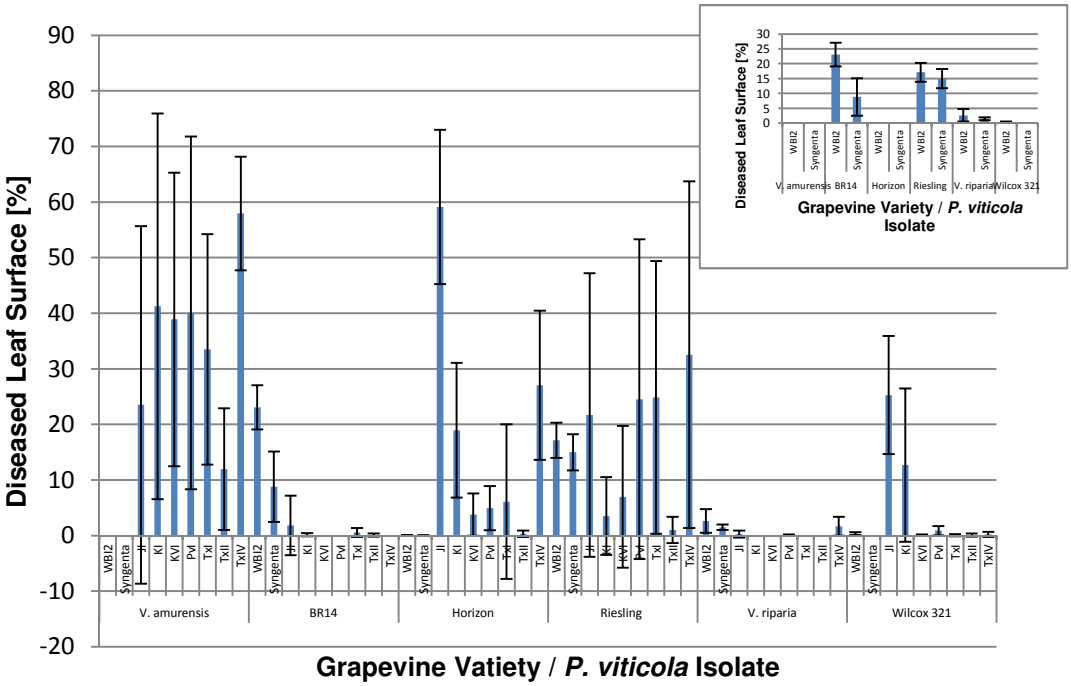


Fig. 17: Average diseased leaf surface of each grapevine variety by *P. viticola* isolates.

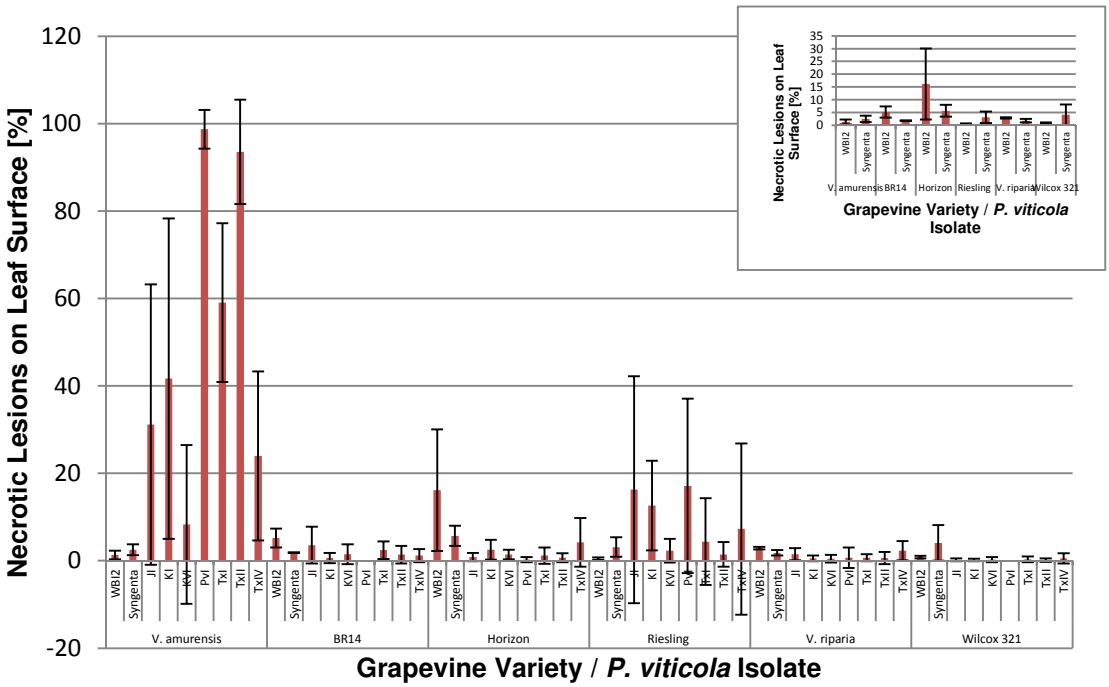


Fig. 18: Average necrotic leaf surface of each grapevine variety by *P. viticola* isolates.

3.1.4. Plasmopara viticola fitness assay

It has been shown, that host defense correlates with downy mildew viability and reproduction (Keil, 2007), and therefore, additionally to cross infections experiments, a fitness assay of *P. viticola* isolates was evaluated.

The ratio of living sporangia out of 100 was counted and the number of zoospores per living sporangium noted.

The viability of the sporangia of each *P. viticola* isolate and their potential to infect different grapevine species is presented in figures 19-20.

Viable *P. viticola* offspring for almost all isolates (50-100% living sporangia, 5-7 nuclei per sporangium) were found on *V. amurensis*. The isolate “TxI” contained an average of 11 nuclei per sporangium. The isolates “MI” and “TxIII” produced only non-viable sporangia. The isolate “Syngenta” contained an average of 6 nuclei per sporangium, but only 12% of them were found to be viable. The isolates “WBI2”, “Syngenta” and “JI”, growing on the grapevine species “BR 14”,

contained 100% living sporangia, with 5-8 nuclei on average. The isolate “TxI” held 27% living sporangia and about 2.5 nuclei on average (Fig. 19 and Fig. 20). For the hybrid grapevine species “Horizon”, the *P. viticola* isolates “WBI2”, “Syngenta” and “TxII” show generally low viability performance (3%, 11% and 28% living sporangia, respectively) and medium number of nuclei (4 nuclei in “WBI2”, 6 nuclei in “Syngenta” and 5 nuclei in “TxII” on average). The *P. viticola* isolates “MI” and “TxIII” show no viability (Fig. 19). All other isolates present a viability ranging from 86 to 100% living sporangia with approximately 6-9 living nuclei. The isolates grown on grapevine cultivar “Riesling” shows viability from 81 to 100%, except the isolate “JI”, with only 31% living sporangia. The sporangia of the isolate “TxIV” contained a reduced number of nuclei (3.3 nuclei on average), whereas all other isolates have at least 4-8 nuclei per sporangium. Except for the *P. viticola* isolate “TxIV” (100% living sporangia and 7.6 nuclei per sporangium), and the *P. viticola* isolate “Syngenta” (2% living sporangia and 2 nuclei per sporangium), there was no reproduction of any other isolate detectable on the wild American grapevine species *V. riparia*.

P. viticola isolates “JI” and “KI”, grown on the grapevine species “Wilcox 321”, show viability of 100% and 91% respectively, and average numbers of nuclei of 4.8 and 6.6, respectively. The viability of the isolate “TxIV” is reduced to 54% living sporangia and 6 nuclei per sporangium. All other *P. viticola* isolates did not produce any viable sporangia (Fig. 19).

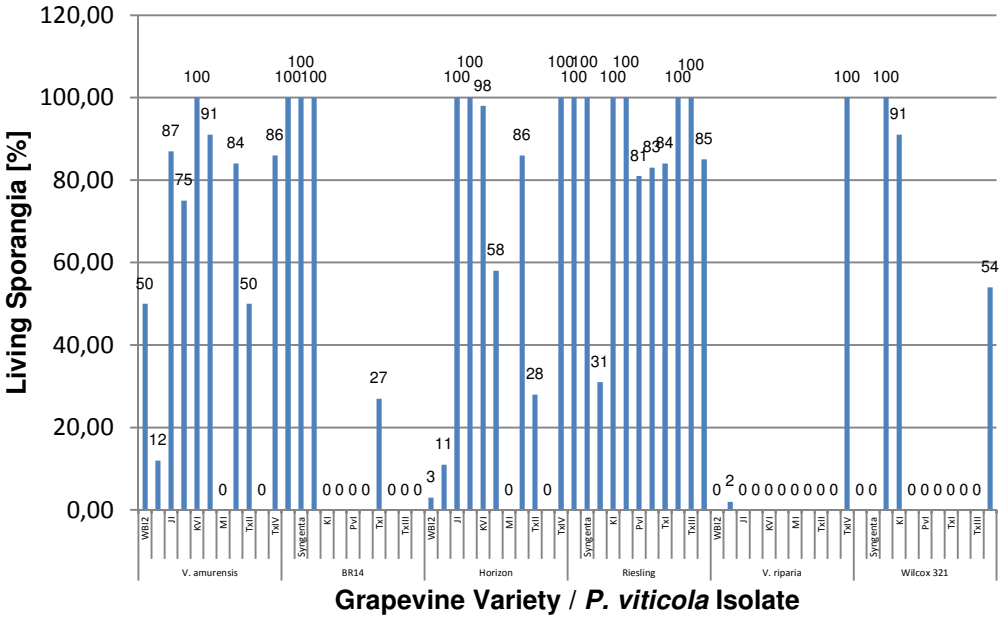


Fig. 19: Average living sporangia [%] of each *P. viticola* isolate, growing on different grapevine species.

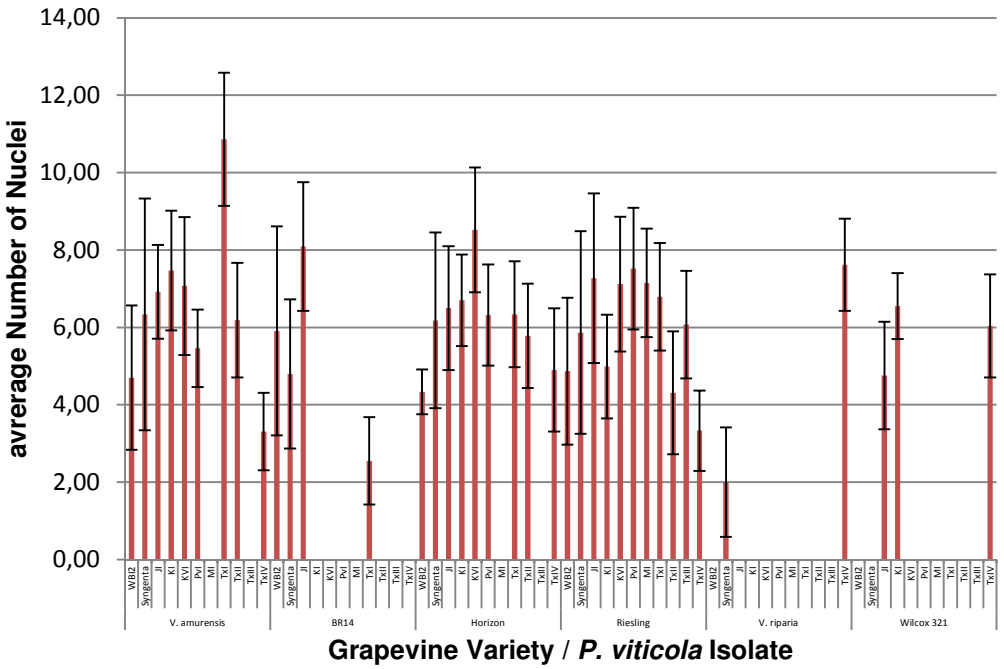


Fig. 20: Average number of nuclei of each *P. viticola* isolate, growing on different grapevine species.

3.1.5. Results of chemotaxis and leaf volatiles assay

Candidates for volatile signals that guide zoospore targeting, were analyzed by Boland *et al.*, (submitted). Leaf volatiles, emitted over three days from cv. "Müller-Thurgau" and from *V. jacquemontii* plants in semi-closed sampling systems were analyzed by gas chromatography/mass spectrometry. Small aldehydes (cis-hexenyl acetate, hexanal, nonanal, and decanal) were detected. These aldehydes have previously been reported by Mendgen *et al.*, (2006) to be bioactive plant volatiles, that are potent regulators of haustoria formation in the obligate biotrophic rust fungi.

The activity of cis-hexenyl acetate, hexanal, nonanal, and decanal in the host-free chemotaxis assay was tested and the spatial distribution, the relation between zoospore frequency and distance from the source were plotted and the regressions determined as measure for the tightness of the correlation. For cis-hexenyl acetate and hexanal, the accumulation of zoospores towards the source was moderate (R^2 = 0.33 and 0.27, respectively) as compared to a piece of cv. "Müller-Thurgau" leaf as a source (R^2 = 0.51), but clearly different from water or a *V. jacquemontii* source (R^2 = 0.12 and 0.11, respectively). In contrast, nonanal as a source was as attractive as an intact cv. "Müller-Thurgau" leaf (R^2 = 0.53), and decanal even exceeded the attractivity of the leaf (R^2 = 0.62), (Boland *et al.*, submitted).

In order to verify nonanal and decanal being bioactive on downy mildew, helping zoospores in stomata sensing, and to test the possibility of controlling infection on a host by an exogenous source of nonanal and decanal, a leaf-disc assay was set up, where leaf discs from susceptible cv. "Müller-Thurgau" were inoculated with either "WBI2" or "Syngenta" *P. viticola* isolate. This was performed in the presence of a small volume of attractants placed on a filter paper in the center of the petridish. The sporulation area was measured in order to evaluate infection success.

The infection of the cultivar "Müller-Thurgau" leaves with *P. viticola* in the presence of potential inhibitors is presented in figure 21.

The results of this assay demonstrate reduced infection for both isolates by nonanal of about 75% on average, whereby already the smallest volume tested (0.01 µl nonanal) was sufficient to produce an inhibition (about 45% on average,

Fig. 21). For decanal (0.01 µl) a similar inhibition was obtained. It was not possible to test even smaller amounts of attractants, because the solvents – required to dilute the attractans – strongly impaired viability of zoospores. For larger amounts of attractant, even stronger inhibition effects could be detected (up to almost 100% inhibition for *P. viticola* isolate "Syngenta" and 0.1 µl nonanal). Thus, it is possible to strongly impair the infectivity of *P. viticola* by a source of exogenous nonanal or decanal.

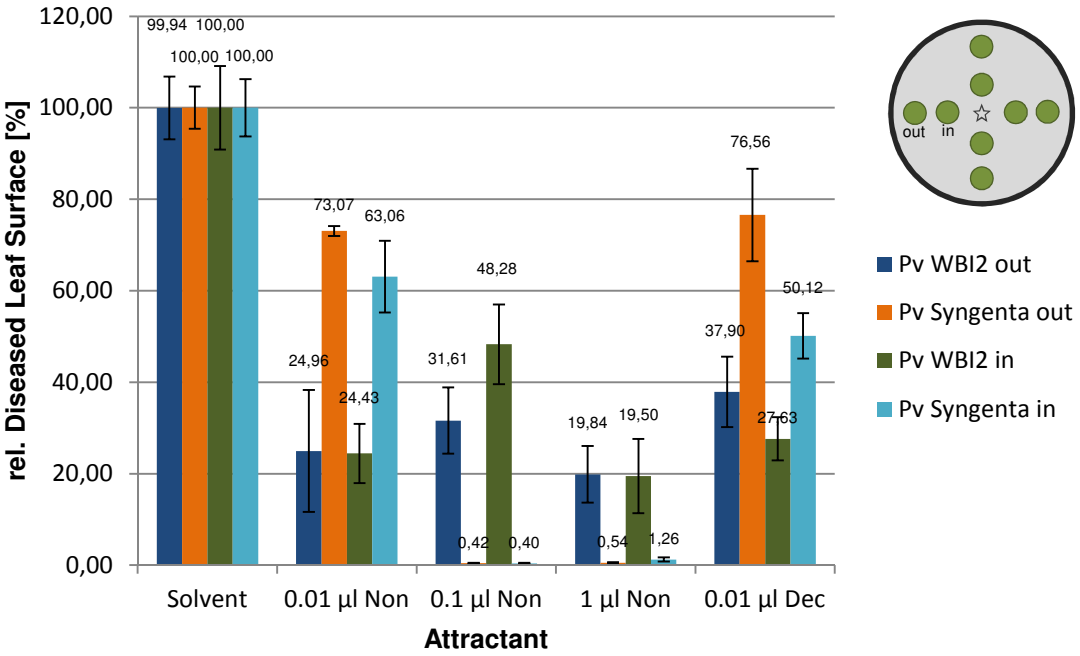


Fig. 21: Inhibition of *Plasmopara viticola* (Pv) isolates WBI2 and Syngenta after treatment with 0.01 µl nonanal, 0.1 µl nonanal, 1µl nonanal and 0.01 µl decanal in relation to solvent (EtOH).

3.1.6. Results of stomata morphology

Previous studies on stomata morphology of *Vitis* species showed that inner stomatal cuticular rims are present in wild *Vitis* species from America and Asia but absent in cultured grapes (Jürges *et al.*, 2009).

Therefore, all grapevine species used for this work were microscopically analyzed to determine the presence of an additional cuticular inner rim and set in relation to cross infection experiments and grapevine species phylogeny.

Results of microscopical evaluation of stomata morphology (presented in table 7 and figure 22) of cultured grapes (*V. vinifera* cv. "Müller-Thurgau" and *V. vinifera* cv. "Riesling") and wild American and Asian species (*V. riparia* and *V. amurensis*) confirmed these results. In contrast, within the hybrids ("BR 14", "Horizon" and "Wilcox 321"), only in "BR 14" inner stomatal rims are present. In the wild European grapevine species *V. sylvestris*, inner rims were present in four species (H13, K39, K53 and K103) and lacking in six species (K15, K20, K83, K94, K95 and K99), (Tab. 7 and Fig. 22).

Grapevine variety	Stomatal Inner Rim
Vitis vinifera cv. Müller-Thurgau	No
Vitis vinifera cv. Riesling	No
Vitis vinifera cv. Regent	No
***Vitis* hybrid BR 14**	**Yes**
Vitis hybrid Horizon	No
Vitis hybrid Wilcox 321	No
Vitis riparia	**Yes**
Vitis amurensis	**Yes**
***Vitis sylvestris* (H13)**	**Yes**
Vitis sylvestris (K15)	No
Vitis sylvestris (K20)	No
***Vitis sylvestris* (K39)**	**Yes**
***Vitis sylvestris* (K53)**	**Yes**
Vitis sylvestris (K83)	No
Vitis sylvestris (K94)	No
Vitis sylvestris (K95)	No
Vitis sylvestris (K99)	No
***Vitis sylvestris* (K103)**	**Yes**

Tab. 7: List of grapevine varieties with or without stomatal inner rims.

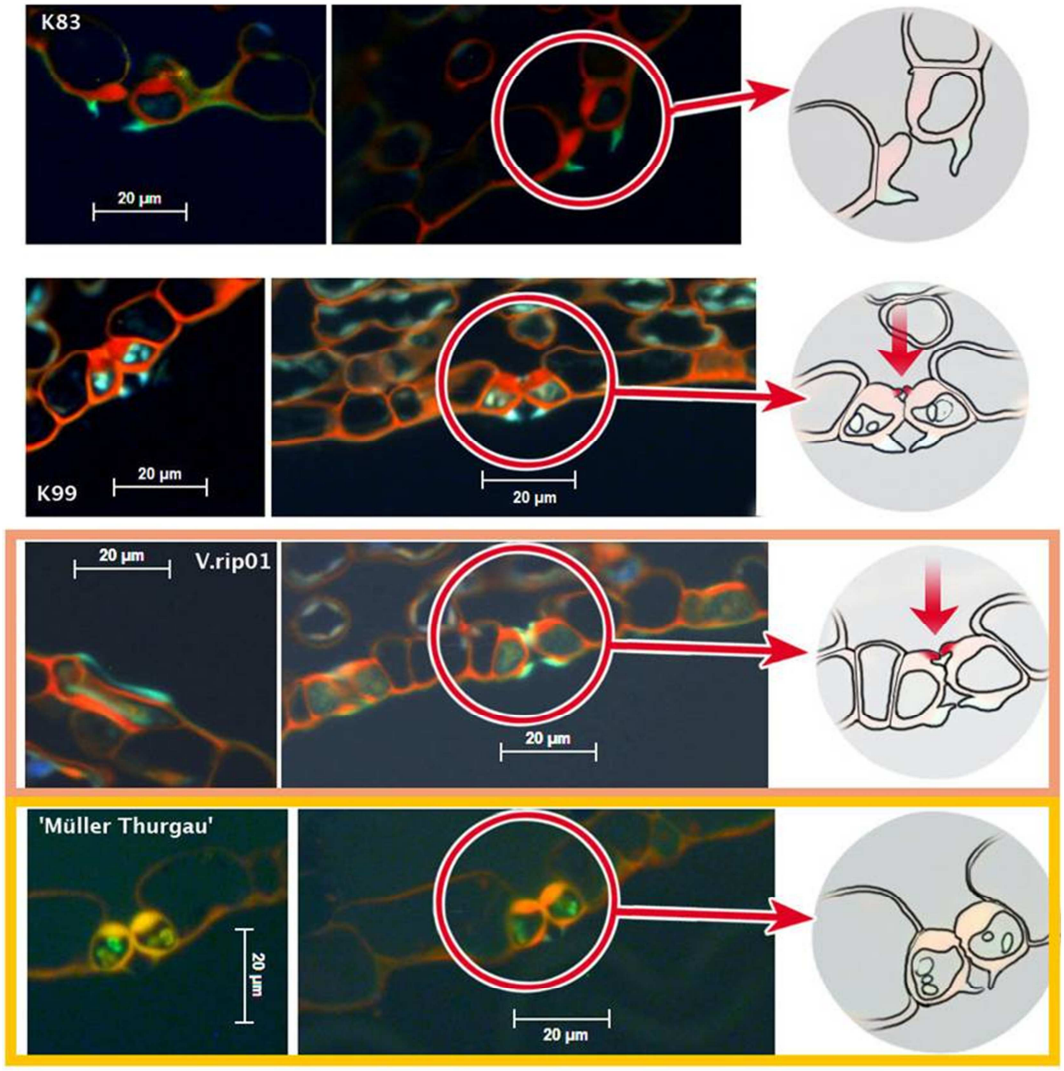


Fig. 22: Microscope pictures of *Vitis sylvestris* K83 (without inner stomatal rim) and K99 (with inner stomatal rim, see red arrow) as well as *Vitis riparia* (inner rim present, see red arrow) and *Vitis vinifera* cv. “Müller-Thurgau” (no inner rim present). Pictures were taken and drawings were made by M. Rühle.

3.2. Genetic diversity of *Vitis sylvestris*

In the second part of this study the naïve host *Vitis sylvestris* was investigated with the main focus laid on a population collected from the peninsula “Ketsch” which is located along the upper Rhine River in Germany.

Genetic diversity and distances were measured and correlated to actual geographic distances.

In order to find preadaptation to the pathogen in *V. sylvestris*, an infection assay was performed with a downy mildew strain from Palatinate, Germany, and 10 different *V. sylvetris* plants. The results were compared to the susceptible cultured grapevine species “Müller-Thurgau”, “Riesling” and to the resistant grapevine species “Regent”.

3.2.1. SSR-marker analysis

To identify the genetic diversity of *Vitis sylvestris*, a SSR-marker analysis was performed using eight different SSR-markers. The results of the analysis are displayed in table 8, (the data for this analysis was partially obtained by I. Valea).

The total number of alleles obtained from 8 loci is 121. The number of alleles per locus ranges from 9 (VrZag79) to 18 (VVMD25) with a mean of 15.125. The estimated values of the expected heterozygosity (H_e), as a measure for genetic diversity of the loci, varied from 0.3961 (VrZag79) to 0.8483 (VVMD32) with a mean of 0.7509. The observed heterozygosity (H_o), representing the percentage of heterozygous individuals among all tested ones, ranges from 0.91803 to 1 with a mean of 0.98771. The estimated frequencies of null alleles are all negative, meaning that all alleles are informative. PI (Probability of Identity, Paetkau *et al.*, 1995) values range from 0.0876 (VVMD25) to 0.4012 (VrZag79), Therefore, VrZAG79 is the least informative SSR marker. The mean of -0.3154 for the F-statistic value F_{is} (Wright, 1965) indicates that inbreeding does not play a role (Tab. 8).

SSR	Observed Number of Alleles	Estimated Frequency of Null Alleles	H_e	H_o	F_{is}	PI
VVS2	17	-0.1150	0.7936	1	-0.2601	0.1063
VVMD07	17	-0.0459	0.8338	0.91803	-0.101	0.0876
VVMD25	18	-0.0898	0.8351	1	-0.1974	0.0856
VVMD27	15	-0.1378	0.7577	1	-0.3197	0.1301
VVMD28	16	-0.1156	0.778	0.98361	-0.2642	0.1371
VVMD32	13	-0.0820	0.8483	1	-0.1788	0.0745
VrZag62	16	-0.1336	0.7642	1	-0.3086	0.1378
VrZag79	9	-0.4325	0.3961	1	-1.5244	0.4012
Mean	15.125		0.7509	0.98771	-0.3154	
Total	121		6.0069	7.90164	-0.3154	

Tab. 8: Statistical results of SSR-marker analysis. H_e: expected heterozygosity; H_o: observed heterozygosity; inbreeding index (F_{is}) and Probability of Identity (PI).

3.2.2. Vitis sylvesrtis dendrogram

The dendrogram of genetic distances between wild *V. sylvestris* grapes collected from “Ketsch”, Germany is presented in figure 23.

The wild European grapevine accessions clustered separately from cultivated grapes as well as from wild *Vitis* species originating from Asia and America (Fig. 23). Two exceptions to this trend are *Vitis sylvestris* species (Oe8 and Oe12) originating from the Danube valley. It can be assumed that they were misidentified as *V. sylvestris*. The *V. sylvestris* species are divided in seven clusters. Cluster 8 and 9 are representing the cultivated grapevine species and the wild American and Asian species, respectively (Fig. 23). Compared to cultured grapes, *V. sylvestris*’ dendrogram shows a higher genetic diversity due to a higher number of alleles per locus (Moncada *et al.*, 2006, Fig. 23).

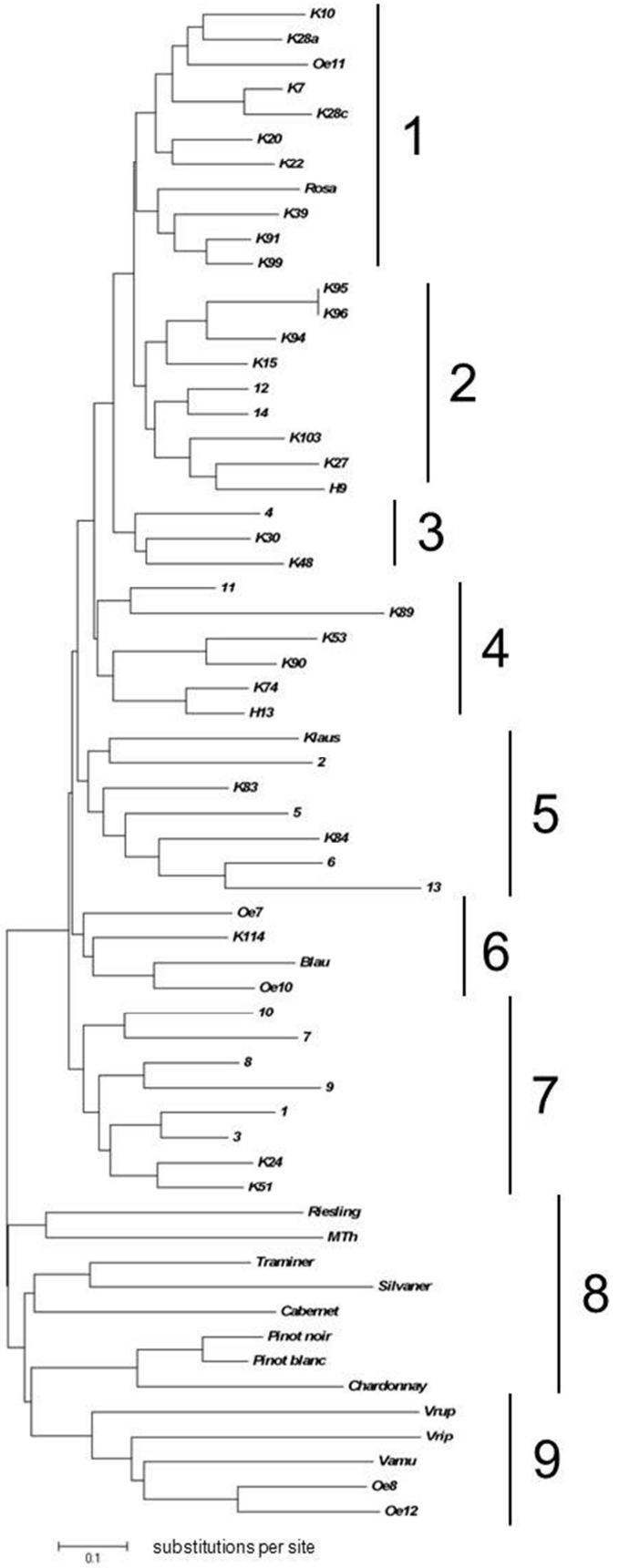


Fig. 23: Dendrogram of *Vitis sylvestris* species.

3.2.3. Distance matrix of *Vitis sylvestris* species at “Ketsch”

In order to visualize the geographic distances of the *V. sylvestris* plants collected at “Ketsch”, Germany, a distances matrix was set up and calculated by Neighbor Joining method. The dendrogram of geographic distances between wild *V. sylvestris* grapes is presented in figure 24.

The distribution of *V. sylvestris* on the peninsula “Ketsch” is clustered. However, this dendrogram can be generally divided in three different groups. Each group divides further into two subgroups (Fig. 24).

The biggest distance between two grapevine species, which is also the biggest distance in north-south direction, is 2.21 km between K102 and K85. The biggest distance in cluster 1 is 0.9 km between K102 and K115, 0.37 km for cluster 2 between K118 and K15 and 1.14 km in cluster 3 between K86 and K85.

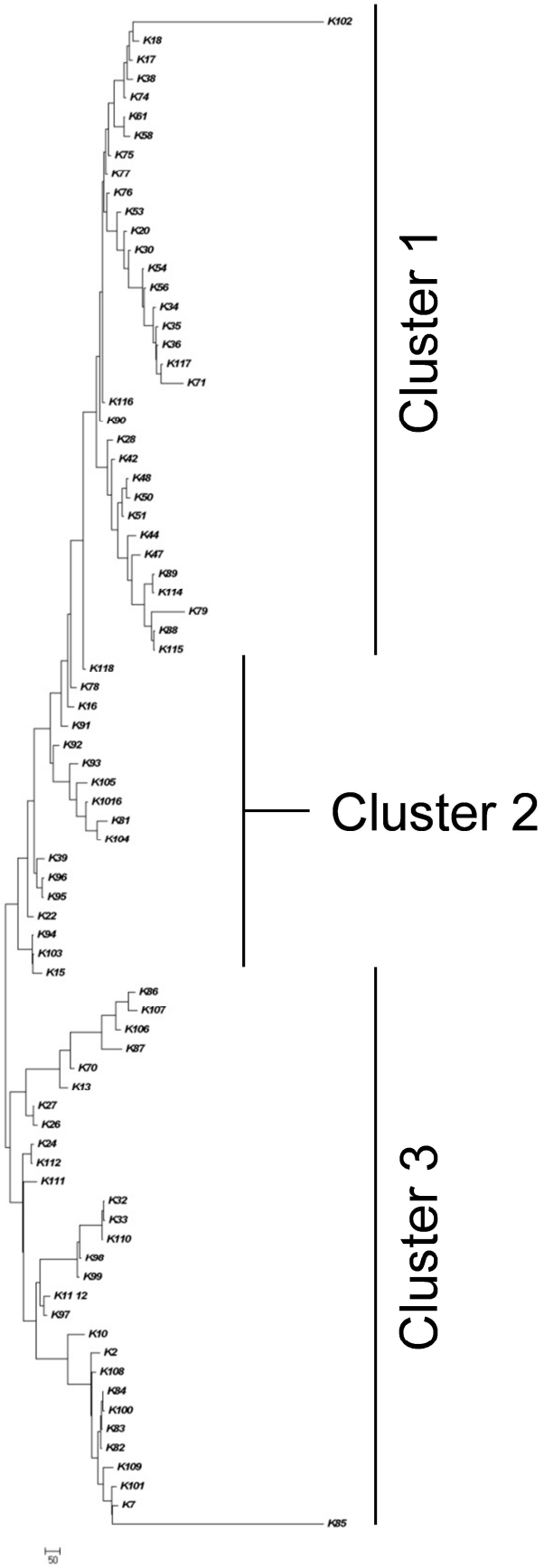


Fig. 24: Dendrogram of geographic distance between each *Vitis sylvestris* accession from “Ketsch”.

3.2.4. Genetic and geographic correlation of wild *Vitis sylvestris* originating from “Ketsch”, Germany

Genetic and geographic distances were set in correlation, in order to determine families within the “Ketsch” population and the consequential gene flow.

The total correlation of genetic and geographical distance between wild *V. sylvestris* grapes is demonstrated in figure 25. The geographic distribution and genetic relationship is correlated with the factor $R^2 = 0.21$ (Fig. 25), the slope of the straight line is 0.0004 as a measurement of the gene flow.

Families found in clusters 1, 2 and 4, have even higher correlation factors according to the *Vitis sylvestris* dendrogram ($R^2 = 0.34$ for cluster 1, $R^2 = 0.48$ for cluster 2 and $R^2 = 0.89$ for cluster 4) (Fig. 23). The slopes of the curves for the three clusters are 0.0004 for cluster 1, 0.0012 for cluster 2 and 0.0023 for cluster 4, meaning cluster 4 has the lowest gene flow.

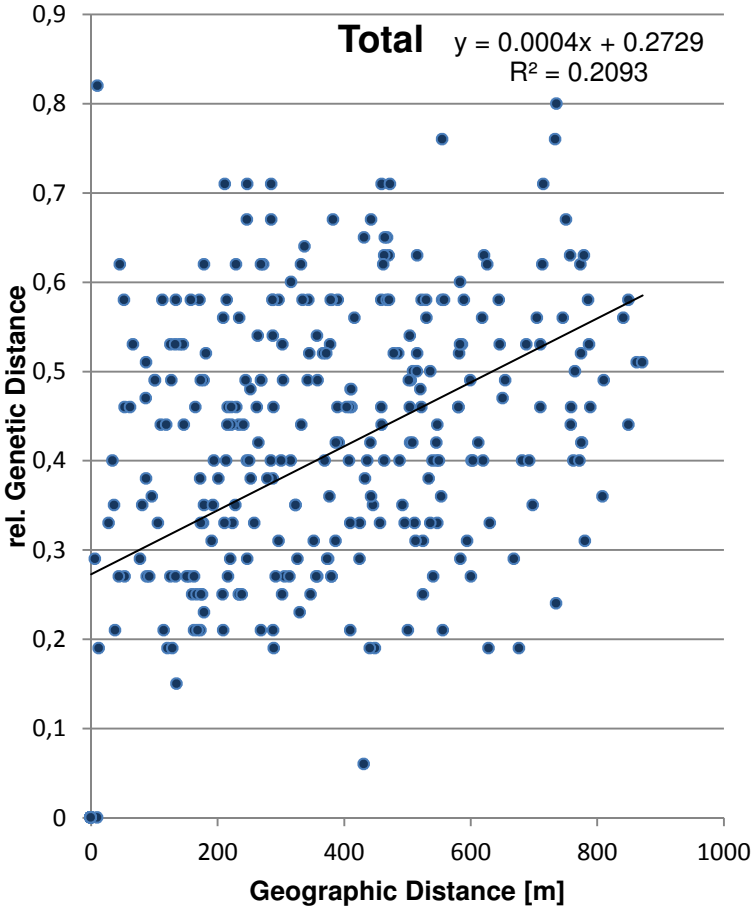


Fig. 25: Correlation between geographic and genetic distance of *Vitis sylvestris* species from “Ketsch”.

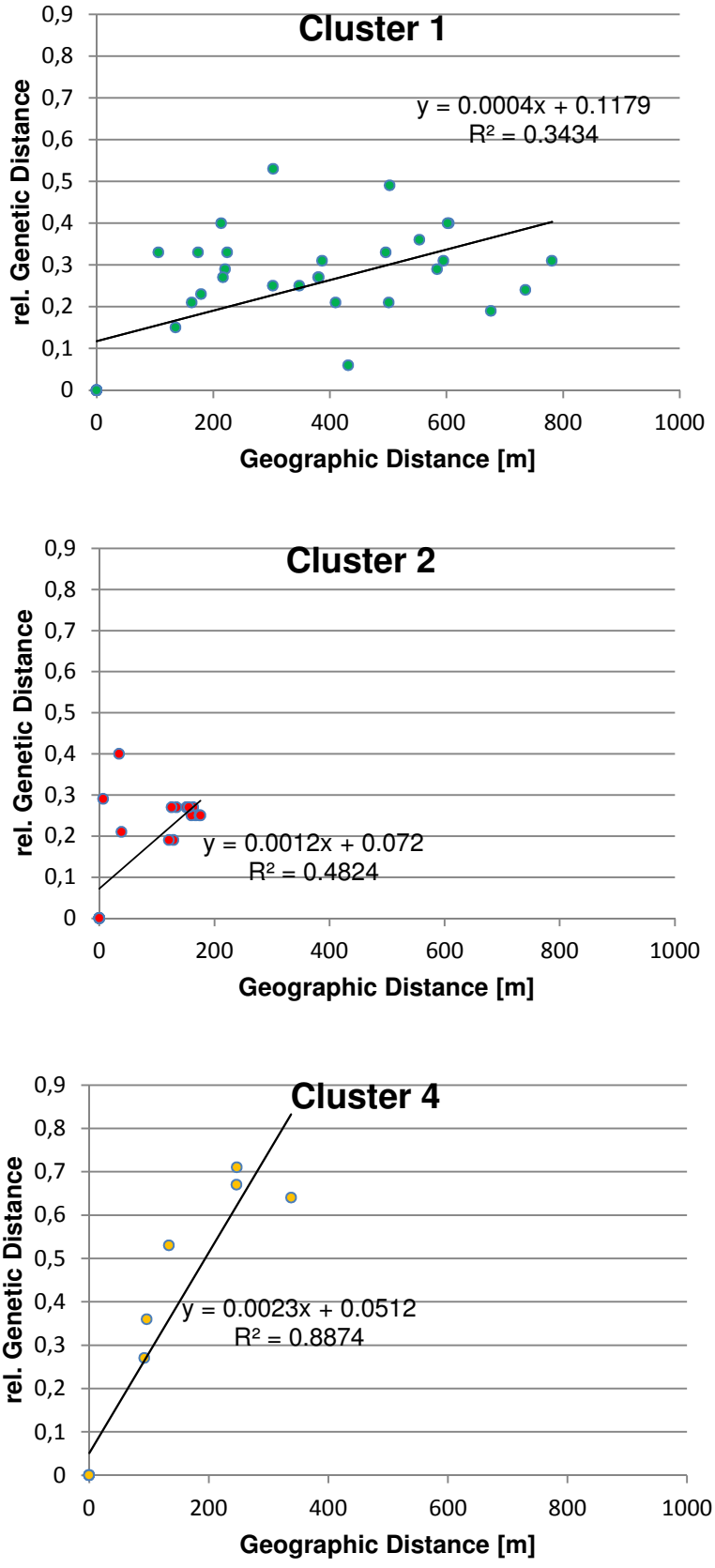


Fig. 26: Correlation between geographic and genetic distance of *Vitis sylvestris* families from "Ketsch".

3.2.5. Results of *Vitis sylvestris* infection assay

To test *Vitis sylvetris* preadaptation to *Plasmopara viticola*, an infection assay was performed (in cooperation with A. Kortekamp, DLR, Dienstleistungszentrum Ländlicher Raum, Breitenweg 71, 67435 Neustadt, Germany).

Detached leaves were sprayed with a spore suspension containing 50 000 sporangiospores/ml and incubated in Petridishes at 21°C in a climate chamber (16h light period, followed by 8h darkness). After an incubation period of 7 days, sporulation intensity was determined by microscopical analysis.

In relation to pathogenic resistance, the wild grape plants tested show significant resistance to the downy mildew population isolated from the Palatinate, which equals to that of the resistant grapevine cultivar “Regent” (Fig. 27). Whereas the cultivar “Müller-Thurgau” shows an average of 78.3% infected leaf surface and the cultivar “Riesling” 71.7%, the so called “resistant” cultivar “Regent” only shows an average of 15% infected leaf surface. Surprisingly, also the wild grapes show a strong resistance to *Plasmopara viticola*. Only the leaf surface of the wild grape K20 shows an average infection of 22.3%, all other grapes show less than 15% of infection. In fact most of them show less than ten percent. For instance K83 shows only 2.3% infected leaf surface on average.

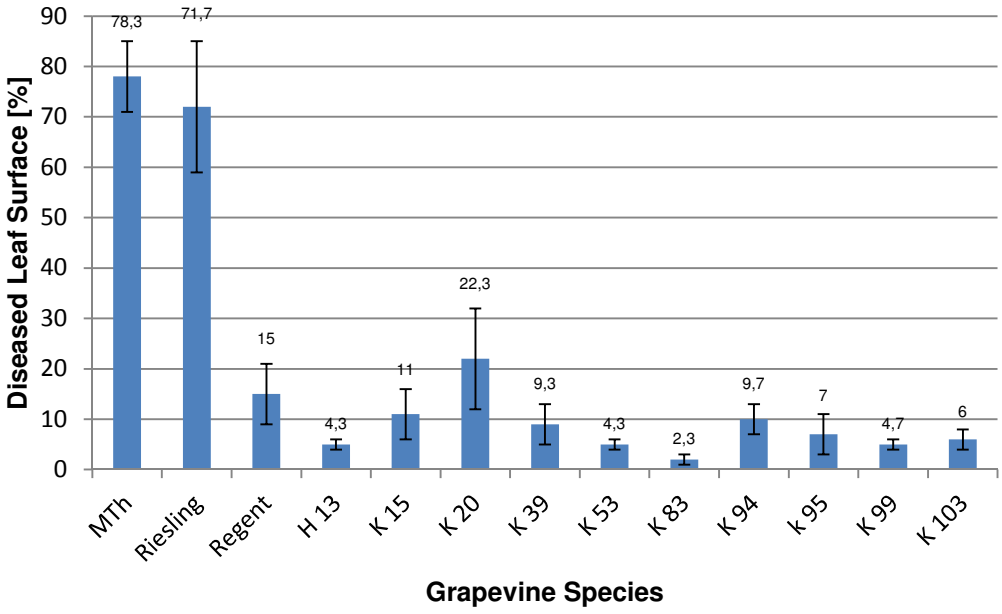


Fig. 27: Diseased leaf surface in [%] for the tested grapevine species.

3.3. Summary of results

In this work it was shown, that there exist more *P. viticola* strains but one, although these stains do not occur in Europe and they also do not show any changes in infection behavior towards *Vitis*.

It was also shown that the zoospores of *P. viticola* can detect the stomata of plants using a nonanal gradient. Some grapevine species, however, emit less nonanal than others and therefore cannot be infected so easily. In the presence of exogenous nonanal the infection rate of the cultivar “Müller-Thurgau” was reduced by an average of 75%.

Further the results showed, that the population of European wild grapevine species *Vitis sylvestris*, originating from the area of "Ketch", Germany, are protected from introgression by cultured grapevine species due to low gene flow on the one hand, on the other hand a sufficient genetic diversity prevents this population from inbreeding.

Additionally, it was shown that the wild grape *Vitis sylvestris* is resistant to downy mildew.

4. Discussion

The American grapevine pathogen *Plasmopara viticola* (downy mildew) was introduced to Europe in the late 19th century, where it devastated many vineyards and brought great economic loss to the grape growers. Nowadays downy mildew is still one of the most dangerous threats to vineyards and can only be controlled by ecologically questionable pest management.

Despite its great economic impact on the grape growing industry and ecological damage caused by fungicides used for pest control, open questions remain in order to fully understand *P. viticola* infection mechanisms and grapevine defense mechanisms respectively:

1. *Plasmopara viticola* phylogeny is still not completely elucidated. The host genus *Vitis* has experienced several stages of geographic isolation. Was this accompanied by a geographic isolation of the parasite? And does this lead to a radiation of this immigrating parasite? What species that are related to *Plasmopara viticola* coexist e.g. in Asia with wild species of *Vitis*, and are they able to infect these potential host species as effective as *Plasmopara viticola* does infect cultured grapes?

2. The *Plasmopara / Vitis* pathosystem provides the unique case that we have a host population which was free of parasite due to of geographic isolation, and that we have a historically recorded immigration of the parasite into this population. It is known that *Vitis vinifera* is susceptible to the pathogen, but what about its ancestor *Vitis sylvertis*? Is there a preadaptation to the newly introduced pathogen?

In the following the results of this work shall be discussed: The first part deals with *P. viticola* phylogeny with respect to the phylogeny of the host and the results of host / pathogen interaction.

The second part considers the naïve host *Vitis sylvestris*, its genetic diversity tested in a population originating from “Ketsch”, Germany, and its preadaptation to *P. viticola*.

4.1. Genotypic identification and comparative analysis of host / pathogen interaction

The first part of this study focused on pathogen and host phylogeny as well as on host specification and the interaction with the pathogen.

4.1.1. Cryptic diversity of *Plasmopara viticola* in North America

For the genus *Vitis*, several varieties of *P. viticola* and the rare East Asian species *P. amurensis* have been described (Săvulescu, 1941, Săvulescu & Săvulescu, 1952, Golovina, 1955). Investigations of Rafailă *et al.* (1968) on the other hand, did not reveal clear morphological differences for *Plasmopara* accessions from the Vitaceae. It thus seems possible that the minor differences observed by Săvulescu (1941), Săvulescu & Săvulescu (1952), and Golovina (1955) are due to environmental conditions (Dudka *et al.*, 2007) or the host matrix, as it has recently been demonstrated, that the host matrix can have a major impact on downy mildew morphology (Runge & Thines, 2010). However, the lack of morphological divergence does not necessarily indicate species identity. The three distinct lineages found in this study show significant genetic divergence (1.0 to 1.4%), which is lower than the divergence of the genera *Novotelnova* and *Protobremia* (1.9%), but comparable to the divergence of other *Plasmopara* species, like *P. obducens* and *P. constantinescui* (1.5%) (Voglmayr & Thines, 2007). It is also significantly higher than in closely related species within the genus, such as *P. densa* and *P. euphrasiae* (Voglmayr & Constantinescu, 2008), which amount to only 0.4 to 0.5%.

The host range of the three cryptic lineages remains unclear. Jürges *et al.* (2009) have shown that European isolates of *P. viticola* do not readily infect *Vitis* spp. from North America, and East Asia. In the former case, strong defence reactions were observed, while in the latter case, surface mycelium without successful entry has been produced, suggesting a high degree of specialisation.

Two of the three lineages have not been found in Europe yet, however, it cannot be ruled out that they were introduced to Europe together with cultivated hybrids of *Vitis vinifera* and the corresponding native hosts.

4.1.2. *Vitis* Phylogeny

For the investigation of *Vitis* and *P. viticola* interaction in subsequent experiments, such as cross infections, fitness assays, and morphological structures, it is necessary to know detailed *Vitis* phylogeny in respect to the *Vitis* species used for these experiments.

Their correct identification and positioning in the phylogenetic tree was carried out by comparing them to preexisting data of phylogenetic analyses with 3 cpDNA regions (two introns, one IGS) of 47 accessions, comprising 29 *Vitis* species and several cultivars of *Vitis vinifera* (Tröndle *et al.*, 2010).

Phylogenetic analysis of the combined data set showed, that the tested *Vitis* accessions split into 3 clades that mostly mirror the geographic distribution of the taxa. For the few cases, where single accessions from other continents cluster into the clade of a different continent, the morphology indicates recent hybridizations between cultivated grapevine and autochthonous species (Tröndle *et al.*, 2010).

For this study six additional *Vitis* species were added to the original phylogenetic tree (Fig. 16, p. 42, marked with an asterisk): *V. amurensis* (VAmu2 *), originating from Asia, *V. riparia* (VRip2 *) from the American continent, *V. vinifera* cv. “Riesling” (CvRies *), originating from Europe, and the hybrids *V.* spec. “BR 14” (CvBR14 *), *V.* spec. “Horizon” (CvHorizon *) and *V.* spec. “Wilcox 321” (CvWilcox321 *).

In this condensed Maximum Parsimony tree, *V. amurensis* (VAmu2 *) clustered with *V. betulifolia* instead of *V. amurensis* species VAmu1. This is due to the low number of base pair differences: *V. amurensis* (VAmu2*) and *V. betulifolia* show only one base pair difference, *V. amurensis* (VAmu2*) and *V. amurensis* (VAmu1) are different in two base pairs. This could be explained by either a point mutation or, even more likely, by a sequencing mistake.

The cultivar “Riesling” clustered with the other European *V. vinifera* species.

Due to the low bootstrap values, most American *Vitis* species cluster in one branch. Therefore, despite of the difference of two base pairs between both *V. riparia* accessions, *V. riparia* (VRip2 *) takes place next to *V. riparia* (VRip1) in the condensed Maximum Parsimony tree.

Also the three hybrids (“BR 14”, “Horizon” and “Wilcox 321”) clustered in this branch. This is not surprising, considering their pedigree. The *Vitis* species “BR 14” is a hybrid from *V. rupestris* and Seibel grape 14664. The hybrid *V.* spec. “Horizon” is related to *V. labrusca* (Reisch *et al.*, 1982, Wellington, 1939) and the hybrid “Wilcox 321” is a *V. riparia* hybrid (Swenson, 1985).

4.1.3. Cross Infections

The cross infection experiments were performed in order to relate pathogen aggressiveness to host specific defense intensity. Six different grapevine species have been tested (*V. amurensis*, *V. riparia* and *Vitis vinifera* cv. “Riesling” and the hybrids “BR 14”, “Horizon” and “Wilcox 321”). The grapevine species were selected according to observed infection severity towards downy mildew [classified by staff members of Plant Genetic Resources Unit (PGRU), Cornell], and divided into “severity classes”. Then, each grapevine species was infected with the *Plasmopara viticola* isolates collected previously.

The species tested in this experiment have either evolved in the presence of *P. viticola* itself (*V. riparia*), or are cross breeds of *Vitis* species with a co-evolutionary background (“BR 14”, “Horizon” and “Wilcox 321”). In case of *V. amurensis*, evolution in the presence of the close relative of *P. viticola* [eventually *P. amurensis* (Grünzel, 1959)] is assumed. In contrast, *V. vinifera* cv. “Riesling” did not co-evolve with *P. viticola*.

All *P. viticola* isolates, which can be divided in three lineages, evolved with American *Vitis* species. However, although they generally showed infection rates according to host co-evolution, there are two isolates, which showed different results. The isolate “TxII” performed poorly on all grapevine species, whereat the isolate “JI” apparently is more aggressive than others. This cannot be explained by co-evolution and the phylogeny of the host, or by the phylogeny of the pathogen, because both isolates cluster in the same clade within the phylogenetic tree.

It might be connected to the fact, that the isolates “WBI2” and “Syngenta” also show different infection success, where the isolate “WBI2” appears more aggressive then the isolates “Syngenta”. The isolate “Syngenta” is reported to

be resistant to the fungicide “Metalaxyl-M” (Kassemeyer, personal communication), and therefore might have lost infection aggressiveness over fungicide resistance, what is also reported for other *P. viticola* isolates (Delmotte, personal communication). It might be possible, that similar reasons, heat resistance for instance, decreased aggressiveness in the isolate “TxII”, originating from a relatively warm region (Texas, US), and the isolate “JI”, originating from cooler region (New York State, US), remained aggressive, where no heat resistance is necessary.

4.1.4. *Plasmopara viticola* fitness test

Viability and reproduction are crucial prerequisites for a successful pathogen infection and colonization of the host. Therefore, viability of the sporangia of each *P. viticola* isolate and their potential to infect different grapevine species was analyzed.

The fitness assay supports the results of the cross infection assay. Additionally to the isolates analyzed in the cross infection assay, isolates “MI” and “TxIII” were analyzed, too. These two isolates did not grow in a detectable manner on any grapevine species tested, but sporangia could be recovered from the cultivar “Riesling”, where about 80% were found alive, with about 6 nuclei per sporangium. Although both isolates are closely related and cluster separately from most other isolates, their next neighbor in the phylogenetic tree – and building a branch with “TxIII” – is isolate “TxIV”, which shows neither viability nor reproduction problems. Interpretation of this result leads to the conclusion, that “TxIII” already underwent heat adaption, as postulated for “TxII” (see chapter 4.1.3), while “TxIV still remained unadapted, but therefore kept its infectiousness.

In general, successful colonization leads to viable offspring, independent of the aggressiveness of a *P. viticola* isolate and its location in the phylogenetic tree. Even if only about 5-10% of the leaf surface showed infection, most isolates had at least 50%, but normally more than 80% viable offspring with about 5 nuclei per sporangia.

Different results were obtained, when plants showed only very mild infections, as it happened for example for isolates “WBI2” and “Syngenta” growing on *V. riparia*. Even though about 3% of the leaf surface was infected, almost no viable offspring could be produced by the pathogen, and the number of nuclei was reduced. Similar but less drastical, results could be observed for “WBI2” and “Syngenta” growing on *V. amurensis,* as well as “TxI” growing on “BR 14”. This leads to the conclusion, that pathogen development is arrested in resistant grapevine species. This observation relates to Jürges *et al.* (2009), where hyphal growth is impaired in resistant grapevine species.

4.1.5. *Plasmopara viticola* targets stomata by sensing nonanal

P. viticola can adjust their development with respect to signals perceived from their respective hosts (Boland *et al.*, submitted). Targeting of zoospores to the stomata is essential for a successful infection, because it allows to place the germ tube in the substomatal cavity near the target cells of the spongy parenchyma that are then invaded by haustoria. The pathogen has been reported to block the regulatory circuits that control guard cells such that the stomata remain open (Allègre *et al.*, 2006).

Zoospores can track stomata very efficiently: Within 5-10 minutes after inoculation in cv. “Müller-Thurgau” they have attached to the guard cells, which is consistent with previous results (Kiefer *et al.*, 2002). In this cultivar, the zoospores encyst almost exclusively adjacent to guard cells (Jürges *et al.*, 2009), indicating that they orient with respect to a guiding signal. In fact, when stomata are forced to close by exposing the petiole to abscisic acid, the stomatal targeting to zoospores is inhibited depending on the dose of the stimulus, abscisic acid (Kiefer *et al.*, 2002). When stomatal targeting is impaired, such as on the Asiatic *V. jacquemontii*, this will result in abortive development on the surface of the leaf and reduced infection success (Jürges *et al.*, 2009). These observations support a model where a volatile signal emitted by open stomata, guides chemotactic movement of zoospores.

Boland *et al.*, (submitted) found proof that nonanal and hexanal, oxidative breakdown products of fatty acids (Foissner & Wasternack, 2002), are involved in this targeting process.

The biological function of C9 aldehydes seems to be related to their antipathogenic function, because they are formed rapidly after disruption of the tissue and show fungicidal activities against *Botrytis cinerea* and *Fusarium oxysporum* at concentrations equivalent to those found in disrupted tissues. C9-aldehydes might thus be helpful to sterilize wounds (Matsui *et al.*, 2006).

Interferece with the presumed volatile gradient generated by stomata in leaves of cv. “Müller-Thurgau” by ectopic sources of nonanal and decanal, demonstrated a clear inhibition of infection success (Fig. 21, p. 49) at very small concentrations. Due to the volatile nature of the attractans, statements on the effective concentration of ectopic nonanal in this assay system cannot be made, but even the smallest volume of nonanal that was technically possible to apply produced a clear inhibition of infection success. This can be interpreted as a result of outcompetition of the innate nonanal gradient by the external source, which means that a gradient of nonanal is not only sufficient, but also necessary for stomatal targeting of *P. viticola* zoospores.

Pathogens that have undergone a longer co-evolution history with their respective host, very often have developed strategies to manipulate or usurp so called pathogen-triggered immunity components (PTI) that are produced by the host in response to a challenge (Jones & Dangl, 2006). According to this model, it would be expected that obligate biotrophs, representing advanced stages of co-evolution, might use C9-aldehydes produced by their host to regulate their own development. There is a long and rich history on the effects of plant volatiles on fungi (Fries, 1973). In particular nonanal has been shown to be a key regulator for spore germination in rust fungi (French, 1985). In fact, the rust fungus *Uromyces fabae* was shown to stimulate the emission of nonanal, decanal, and hexenyl acetate from its host, *Vicia faba* L. (Mendgen *et al.*, 2006). When these volatiles were administered to developing spores on an artificial surface, it could be demonstrated that they promoted haustoria development, the central structures for a biotrophic life style. This example implies an interesting regulatory aspect – since the hydroperoxide substrates used by

hydroperoxide lyase are also substrates for allene oxide synthase, the key enzyme of jasmonate synthesis. Furthermore, since there is evidence that the different pathways are regulated by substrate availability (Foissner & Wasternack, 2002), upregulation of the hydroperoxide lyase by the pathogen would be expected with a reduced synthesis of jasmonates (Boland *et al.*, submitted).

The use of small aldehydes to interfere with stomatal targeting of this pathogen has the potential for such a subsidiary strategy.

4.1.6. Stomata morphology of *Vitis* species

Jürges *et al.* (2009) showed that guard-cell morphology is connected to the interaction between *Vitis* and *Plasmopara viticola*. According to Jürges *et al.* (2009), true resistance of *Vitis* species correlates with the presence of an additional cuticular rim at the inner side of the guard-cells, which are present in American and Siberian *Vitis* species.

In this study the stomatal structure was therefore analysed. The microscopical results of cultured grapes and wild American and Asian species confirmed the results reported in Jürges *et al.* (2009). For the hybrids tested ("BR 14", "Horizon" and "Wilcox 321"), only in BR14 inner stomatal rims were found. Obviously, the hybrid "Wilcox 321" did not inherit its ancestors (*V. riparia*) characteristics.

Also the wild European grapevine species *V. sylvestris* was analysed. Inner rims were present in only four species (H13, K39, K53 and K103) and lacking in six species (K15, K20, K83, K94, K95 and K99), (Tab. 7 and Fig. 22, pp. 50-51). This shall be discussed in 4.2.3. (see below).

4.1.7. Conclusions: Genotypic identification and comparative analysis of host / pathogen interaction

Considering the side of the pathogen, three new *Plasmopara viticola* strains were found and their phylogeny determined. Two strains are relatively closely

related and are originating from the State Texas, US, and one strain is originating from New York State, US. All other *P. viticola* isolates, independent of the collection site, were found in another clade of the phylogenetic tree. The infection abilities of these new strains do not differ drastically or in any significantly measurable way from the *Plasmopara* isolates from the main clade. They also do not show a different host preference than the other isolates; leading to the conclusion, that phylogeny of the host does not correlate with the phylogeny of the pathogen on this level, as presumed for a putative pair *V. amurensis* and *P. amurensis*. Measurable differences between single strains were connected to characteristics, not relating to their phylogenetic taxonomy, but features like fungicide or, presumably, heat resistance.

The host, on the other hand, emits nonanal, which attracts *P. viticola* and guides the zoospores towards the stomata of the grapevine leaf. Apparently different host species emit different nonanal concentrations (Boland *et al.*, submitted). Further research has to be done to confirm these preliminary experiments. Next to the development of cuticular inner rims, which are found in resistant grapevine species, and which seem to be sufficient for pathogen defense, the reduction of nonanal emission from the stomata would be another “co-evolutionary” step of host and pathogen interaction in the pair *Vitis* and *P. viticola*.

4.2. *Vitis sylvestris*: Genetic diversity and preadaptation

In the first part of the study, the interaction of different *Plasmopara viticola* isolates with different *Vitis* species was tested and specific resistance patterns of the host against the pathogen were elucidated.

The second part focused on a more detailed level by investigating a specific population of the naïve host *Vitis sylvestris* in terms of genetic diversity and its adaption to the European lineage of *P. viticola*, which was described in 4.1.1.

4.2.1. Genetic diversity of *Vitis sylvestris*

The wild European *Vitis* species, *Vitis sylvestris*, is highly endangered by extinction due to habitat loss (Arnold *et al.*, 2005). Only small populations, or – in some areas – even only single plants, are spread all over Europe (Arnold *et al.*, 1998). The *Vitis sylvestris* population from the upper Rhine River ("Ketsch" peninsula, Germany), is one of the largest in Europe (Ledesma-Krist, personal communication) and up to now there was no study testing introgression and genetic diversity within this population.

Based on a marker set of 8 SSR markers, 50 *Vitis sylvestris*, 8 cultured grapevine species and three wild *Vitis* species from the American and Asian continent respectively, were analyzed (Thomas & Scott, 1993; Bowers *et al.*, 1996, Bowers *et al.*, 1999; Sefc *et al.*, 1999).

The cultured grapevine species and the American and Asian wild *Vitis* species cluster separately from the European wild *Vitis* species, meaning the genetic markers were suitable for a successful discrimination of each species. The appearance of two *Vitis sylvetris* species (Oe8 and Oe12) from the Danube valley, Austria, next to the wild Asian species *Vitis amurensis*, in the dendrogram (Fig. 23, p. 54), is therefore due to misidentification.

The *Vitis sylvetris* species showed a strong genetic diversity (H_e = 0.7509, Tab. 7), which is also represented in the dendrogram (Fig.23), where they are divided in seven clades.

There was evidence for a geographic and genetic correlation between single *Vitis sylvestris* species originating from “Ketsch”, and even more drastically within the single clusters, resulting in a low gene flow.

Within this population, the three Austrian *Vitis sylvestris* species and the one from “Hördt”, Germany, blend into the dendrogram, suggesting no correlation between genetics and geography outside this population. For *Vitis sylvestris* populations originating from Hungary and Turkey a correlation between genetics and geography was assumed (Bodor *et al.*, 2010).

No hybrids could be detected, neither *Vitis vinifera* / *Vitis sylvestris* hybrids, nor hybrids from *Vitis sylvestris* and wild American or Asian species, leading to the conclusion that this population from “Ketsch” is protected from inbreeding, but also from a gene flow from geographically more distanced cultured grapes.

4.2.2. European Wild Grapes - Genetic Relations and Susceptibility to Fungal Pathogens

Vitis sylvestris was commonly considered as susceptible to the pathogen *Plasmopara viticola*. Only in the work of Jürges *et al.* (2009) a certain resistance to the pathogen was described.

Wild European *V. sylvestris* accessions from “Ketsch” clustered separately from cultivated grapes, suggesting that introgression by cultured grapes from geographical closely located vineyards does not play an important role.

This study revealed that *V. sylvestris* is resistant to *P. viticola* to the same degree of the resistant grapevine cultivar “Regent”.

There is no evidence of a correlation between certain clusters of Wild European grapevines and their pathogen resistance to *Plasmopara viticola*.

These findings are surprising, since these presumably autochthonous, European wild accessions, did not co-evolve with the American pathogens and therefore an induced resistance is not very likely (Schröder *et al.*, in preparation). Even though the response of these wild grapes towards several strains of the pathogen isolated from different locations, has to be tested further in order to confirm their obvious resistance features.

Additionally, the permanent threat to wild *Vitis silvestris* species, caused by resistant rootstocks and cultured grapevines in its close neighborhood (Arrigo & Arnold, 2007), could have induced strong selection pressure on this variety,

such that only a few resistant plants, which build an autochthonous, monophyletic group survived and flourished in this area.

4.2.3. Conclusion: *Vitis sylvestris* – Genetic diversity and preadaptation

Genetic distance of wild *Vitis silvestris* species originating from the peninsula "Ketsch" has been shown. It revealed that they are building an autochthonous, monophyletic group. They are clearly differentiating from cultured grapes as well as from wild Asian and American grapevine species. Although their marker variability seems to be higher than from cultured grapes, this study showed that all of them belong to the species *Vitis silvestris*. Due to this genetic diversion and their relatively enclosed geographic location, considering their genetic relationship, a genetic "stability" and therefore a prevention of inbreeding is secured. The wild *Vitis silvestris* from "Ketsch" also showed a considerable resistance to false downy mildew. The resistance response of these grapes was not determined in detail. However, the resistance response against the pathogen will be investigated in the near future using microscopical, biochemical, and molecular techniques. It has to be explained why some *V. sylvestris* species possess cuticular inner rims and some do not. Especially considering that there is no difference in their resistance to downy mildew. Also resistance genes have to be tested. It might be possible, that they do not overlap with resistance genes tested in other resistant grapevine species. In conclusion: The protection of this endangered species is not only highly valuable for sustaining an intact ecosystem, but also for breeding purposes to enhance plant resistance of cultivated grapevine varieties in Europe.

4.3. Outlook

The importance of understanding the pathosystem *Vitis* / *Plasmopara viticola* is based on economic and environmental factors.

In this work it could be demonstrated that three *P. viticola* strains exist, but only one of them occurs in Europe. However, it cannot be ruled out that they were introduced to Europe together with cultivated hybrids of *Vitis vinifera* and the corresponding native hosts. It will be important to determine in future studies, how widespread the cryptic species parasitic to *Vitis* are and whether they pose a potential threat to cultivated grape, in which case quarantine regulations should to be adopted to hinder their further spread.

Additionally, the aldehyde nonanal was found to be bioactive on *P. viticola* hampering the targeting towards guard cells of *Vitis vinifera*. The fact, that exogenous nonanal reduces the infection of the cultivar “Müller-Thurgau” by an average of 75%, opens up the possibility to design new fungicides, which are less invasive to the environment.

The crops wild relative *Vitis sylvestris*, which is highly endangered by introgression from *Vitis vinifera* and other naturalised rootstocks that originate in viticulture, was found to be resistant to *P. viticola*. This finding can lay a base for future breeding programs, due to the fact that *V. sylvestris* is the closest relative to the cultured grapevine species *V. vinifera*. Further tests on this subject will be done using microscopical, biochemical, and molecular techniques, such as QTL (Quantitative Trait Loci) analysis.

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7. Appendix

Accession numbers of *Vitis* species:

Grapevine variety	**intergenic spacer *trnL-trnF***	**tRNA-Leu (*trnL*), intron**	**tRNA-Lys (*trnK*), intron**
***V. vinifera* cv. "Riesling"**	HQ385424	HQ385430	HQ385436
***V.* spec. "BR 14"**	HQ385427	HQ385434	HQ385440
***V.* spec. "Horizon"**	HQ385428	HQ385433	HQ385439
***V.* spec. "Wilcox 321"**	HQ385429	HQ385435	HQ385441
V. riparia	HQ385426	HQ385432	HQ385438
V. amurensis	HQ385425	HQ385431	HQ385437

Accession numbers of *Plasmopara viticola* isolates:

Pathogen	**strain number**	**GenBank accession number *cox2***	**GenBank accession number *ypt1***	**GenBank accession number *nrLSU***
Plasmopara viticola	WBI 1	HM628745	HM628736	HM628765
Plasmopara viticola	Syngenta	HM628744	HM628737	HM628769
Plasmopara viticola	JI	HM628751	HM628732	HM628763
Plasmopara viticola	KI	HM628752	HM628729	HM628766
Plasmopara viticola	TxI	HM628748	HM628734	HM628762
Plasmopara viticola	TxII	HM628746	HM628733	HM628767
Plasmopara viticola	WBI 2	HM628750	HM628730	HM628768
Plasmopara viticola	PvI	HM628749	n.a.	HM628764
Plasmopara viticola s.l.	TxIII	HM628754	HM628728	HM628771
Plasmopara viticola s.l.	TxIV	HM628756	HM628735	HM628770
Plasmopara viticola s.l.	MI	HM628757	HM628731	HM628772

Hiermit erkläre ich, dass ich die vorliegende Dissertation, abgesehen von der Benutzung der angegebenen Hilfsmittel, selbständig verfasst habe.

Alle Stellen, die gemäß Wortlaut oder Inhalt aus anderen Arbeiten entnommen sind, wurden durch Angabe der Quelle als Entlehnungen kenntlich gemacht.

Diese Dissertation liegt in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vor.

Karlsruhe im Oktober 2010

Publikationen

Tröndle, D., Schröder, S., Kiefer, C., Kassemeyer, H.-H., Koch, M., Nick, P., 2010: Molecular Phylogeny of the Genus Vitis based on Plastidic Markers. Am J Bot 97: 1168-1178.

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