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Molecular evidence of intraclonal variation and implications for adaptational traits of grape phylloxera populations (*Daktulosphaira vitifoliae*, Fitch)

Molekularbiologischer Nachweis intraklonaler Variation und Auswirkungen auf die Adaptation bei Reblauspopulationen (*Daktulosphaira vitifoliae*, Fitch)

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Terminology and Abbreviations

16SrDNA	small subunit of ribosomal DNA for procaryotes and chloroplasts
18SrDNA	small subunit of ribosomal DNA for eucaryotes
AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
bp	base pair
cDNA	complementary DNA
cy5	cyanine 5t'-conjugated (labelling of oligonucleotides)
d	days
d.f.	degrees of freedom
ddH_2O	bidest water
DNA	Deoxyribonucleic acid
DNS	Desoxyribonukleinsäure
dNTP	2t'desoxynucleotidetriphosphate
EcoRI	restriction enzyme
e.g.	"exempli gratia" (latin: for example)
EtOH	ethanol
F_{IS}	measure for degree of heterozygosity (F-Statistics)
FISH	Fluorescent *In Situ* Hybridization
F_{ST}	Fixation index (F-Statistics)
FT	Farbteiler (Mikroskopie)
g	gravitation
HWE	Hardy-Weinberg-Equilibrium
LP	Langpassfilter (Mikroskopie)
mA	milli Ampere (10^{-3}Ampere)
mM	millimolar (10^{-3}molar)
MseI	restriction enzyme
ng	nanogram (10^{-9}gram)
NotI	restriction enzyme
p	probability
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pM	picomolar (10^{-12}molar)
RAPD	Random Amplified Polymorphic DNA
rpm	rotations per minute
RT	root temperature
sec	seconds
SQ	Sum of Squares
SSR	Simple Sequence Repeat
Taq	*Thermophilus aquaticus*
U	unit
UPGMA	uUnweighted Pair-Group Method using Arithmetic Averages
UV	ultraviolett
μg	microgram (10^{-6}gram)
μl	microliter (10^{-6}liters)
μm	micrometer (10^{-6} meters)
% v/v	percent volume/volume
%w/v	percent weight/volume
řC	degrees Celsius

1 Introduction

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) are gall-forming aphids native to North America feeding on grapevine (*Vitis* spp. L.). The insect pest was inadvertently introduced into Europe in the 1850s and its feeding on the highly susceptible roots of European grapevines (*Vitis vinifera* L.) led to a fast and widespread destruction of vineyards. Grape phylloxera was subsequently passively dispersed via infested plant material to other wine growing regions worldwide. Finally, the development of resistant rootstocks from native North American species allowed European viticulture to redevelop. The insect pest, however, was not completely eradicated from European vineyards. Today, grape phylloxera populations still persist in specific niches as abandoned vineyards, in which rootstocks proliferate freely and rootstock nurseries, which therefore need intensive insecticide treatments.

Grape phylloxera causes damages on leaves and roots of its host plant. The induced galls are continuously stimulated by injection of saliva and galls thus become strong physiological sinks, notably changing the metabolic activity of the host plant, by accumulating starch. Galled leaves may be damaged through fast dessication (the name phylloxera derives from greek phyllon = leaf and xeros = dry). Roots are even further damaged, when secondary infections arise after the penetration of soil born pathogens into the feeding site. Grafting combines the resistant green part of the *V. vinifera* scion with the resistant roots of American *Vitis* species and thus protects the plant from phylloxera infections. The so-called resistances, however, have been shown to be defeasible and were overcome several times in the past decades. The decline of vineyards as a result of this pest and consequently losses of yield and quality of grapes have been frequently reported in commercial vineyards today (Boubals 1994, Granett *et al.* 1985, Kocsis *et al.* 2002). Approaches to develop alternatives in biological plant protection against phylloxera were recently made by testing the impact of *Metarhizium anisopliae*, soil-born bacteria, which revealed to be very efficient on phylloxera-susceptible rootstocks (Kirchmair *et al.* 2004).

Since its first introduction into Europe a hundred and fifty years ago, *D. vitifoliae* populations seem to have rapidly diversified within European viticultural regions. The high genotypic diversity monitored among these populations (Forneck *et al.* 2000, Vorwerk & Forneck 2006) and the rapid population growth during the summer months allows genotypes to quickly adapt to new environmental conditions or habitats and reveals the high potential for adaptation of this species. A first example of the development of new grape phylloxera biotypes and the failure of phylloxera management by grafting was the detection of biotypes overcoming the resistance of AxR#1 rootstocks in California in the 1960s (Granett *et al.* 1985). Genetic monitoring of populations employing new codominant markers (Corrie *et al.* 2002, Vorwerk & Forneck 2006) and the use of near-natural bioassays (Ritter *et al.* 2007) will allow to gain further information on these rapidly developing insect pest and its adaptation to new hosts.

The insect's life cycle similar to the rapid changes in insect-host plant interactions is not as fixed as it was described by Fitch (1855) and others in the 19^{th} century. Meanwhile a range of life cycle variations has been described (Corrie *et al.* 2002, Downie & Granett 1998, Granett *et al.* 2001). Recent studies revealed that grape phylloxera populations mainly reproduce asexually (Corrie *et al.* 2002, Vorwerk & Forneck 2006).

Asexual reproduction is commonly known from prokaryotes and clones here refer to groups of genetically identical individuals. The asexual reproduction of higher organisms, however, has only recently become an important field of research (Gill *et al.* 1995).

In agricultural research, aphids provide an excellent example for eukaryotic organisms, having adapted at least partially to clonal reproduction. They are able to produce enormous numbers of offspring by parthenogenesis in short time and secure the continuity of their populations by an alternation of seasonally-based asexual and sexual generations (Dixon, 1998, Simon *et al.* 2002). However, exact genetic identity is probably rarely, if ever, maintained for long. It seems improbable that asexually reproducing eukaryotes, which need to transfer exact replica of their whole genome to the offspring, may remain identical copies over several generations. This has already been demonstrated for some aphid species in a series of molecular marker studies (De Barro *et al.* 1994, Field & Blackman 2003, Lushai *et al.* 1997, Lushai *et al.* 1998, Lushai & Loxdale 2003). Genetic variation may be caused by mutations, transposable elements, symbiotic microorganisms or host alternations. Genetic variation within a group of clonally produced individuals is termed "intraclonal variation". Intraclonal variation may not be directly seizable in the phenotype and needs to be detected by molecular methods.

Grape phylloxera represents an ideal organism to analyse the mechanisms of intraclonal variation. This major agricultural insect pest reveals parthenogenetic reproduction in the field and can be easily maintained under constant climatic conditions in the greenhouse or *in vitro*. The insect's fast reproduction makes it possible to analyse several asexual generations within a short time period. Since it is non-host-alternating and does not transmit any viral material, the genetic analysis may be easily performed compared to other aphid species.

This thesis aimed at capturing a range of aspects on the subject of intraclonal variation. First the predominant reproductive mode was to be assessed for European grape phylloxera field populations. Employing new codominant markers, the genotypic structure of grape phylloxera populations was evaluated within and among habitats and over time. Detailed analysis of intraclonal variation was performed on parthenogenetic lineages of grape phylloxera reared in isolation chambers in the greenhouse. Intraclonal genetic variation and characteristics of mutations were analysed using a multilocus marker system. The same marker system was applied to a second aphid species, *Sitobion avenae*, for control and comparison of genetic data. Genetic variation in grape phylloxera deriving from contaminating templates of plant or bacterial DNA were excluded as a source of genetic variation, although a bacterial species was shown to be constantly associated with *D. vitifoliae*. The genetic observations

of intraclonal variation were further combined with an examination of physiological performance data in order to evaluate adaptational traits of asexual grape phylloxera.

2 Literature Review

2.1 Organism of study

Introduction to grape phylloxera

The dwarf aphid *Daktulosphaira vitifoliae* (Fitch) belongs to the family of Phylloxeridae, close relatives of the Aphididae. Phylloxeridae feed on the roots and green parts of woody plants such as grapevine, willow, chestnut, oak, pear, pecan and hickory (Blackman & Eastop 1994). Their name derives from phyllon (greek: leaf) and xeros (greek: dry), which points to the visible damage caused by galling reactions on leaves of grapevine of the genus *Vitis* (L.) (Davidson & Nougaret 1921).

Scientists became involved with the nature of the insect, after its introduction into Europe in the 1850s, when it rapidly destroyed over two thirds of the European vineyards and caused severe economic damage (Fitch 1855, Shimer 1867). Several synonymies thus exist, as e.g. *Viteus vitifolii* (Shimer), *Dactylosphaera vitifoliae* (Shimer), *Daktulosphaira vitifoliae* (Fitch), *Pemphigus vitifoliae* (Fitch), *Phylloxera vastatrix* (Planchon) and *Phylloxera pervastatrix* (Börner), the latter being introduced by Börner (1914) as a distinct species due to morphological differences. Actual publications mainly agree on employing the name *Daktulosphaira vitifoliae* (Fitch) (Russell 1974).

Due to its fast spread, not only in Europe, but in most winegrowing regions worldwide, the insect became notorious in over 2000 articles, revised by Galet (1982). It was only confined by the development of resistant rootstocks, which could not be attacked by the insect. By grafting American *Vitis* species to susceptible European *V. vinifera*, the insect pest has now successfully been controlled for many decades - probably one of the most effective long-term examples of a biological control mechanism adopted for an important agricultural pest.

Life cycle

The common life cycle of the insect was described by Fitch (1855), Shimer (1867), Cornu (1873), Balbiani (1874) and Riley (1876) to be cyclical parthenogenetic. The fundatrix, hatching from the sexually produced overwintering egg, initiates the parthenogenetic leaf galling population in spring. Parthenogenetic reproduction replicates the population multiple-fold throughout the summer, each individual laying up to 300 eggs. Six to seven generations occur on leaves throughout the summer. At the end of the season, dimorphic forms are developed, producing sexual forms, which mate and lay a single overwintering egg. The fundatrix hatching from this egg will then initiate the parthenogenetic cycle of the following year.

The life cycle, as described here, resembles that of many members of the Aphidoidea. Most Aphidoidae shift to a secondary host during summer season, where parthenogenetic reproduction occurs. Grape phylloxera do not alternate hosts, sexual and asexual life cycle

are completed on roots and leaves of the same plant.

Variations of life cycle

This now commonly accepted life cycle of grape phylloxera, however, has been shown to be variable in several situations, depending on the environment and *Vitis* species colonised. Downie & Granett (1998) reported the absence of winged or root-feeding forms and the completion of the sexual cycle within leaf galls on *Vitis arizonica* in Southwestern USA. Complete absence of the sexual cycle and therefore putative parthenogenetic populations of grape phylloxera were observed in California (Granett *et al.* 2001), although obligate parthenogenetic reproduction has not been confirmed yet. In addition, recent studies employing molecular markers on grape phylloxera populations in Australia revealed, that the classical life cycle does not apply for these populations, but that parthenogenesis is the predominant mode of reproduction in most infected vineyards there (Corrie *et al.* 2002). Although European grape phylloxera show the ability to reproduce sexually under laboratory conditions (Forneck *et al.* 2001), the completion of the sexual cycle in the field has not been confirmed in recent years. Only few single field-sampled sexual stages were documented in the studies of Stellwaag (1928) and Balbiani (1975). Sexual recombination in European grape phylloxera populations was presumed to occur rarely and discussed to be dependent on climatic factors (Forneck *et al.* 2001). However, these observations were based on AFLP markers, which only reveal dominant inheritance patterns and make it difficult to determine events of recombination and meiosis. Studies, employing codominant markers, as shown with new microsatellites in this thesis (Vorwerk & Forneck 2006, see chapter 3) were needed to re-examine the question for European grape phylloxera populations.

2.2 Clonality

Parthenogenesis, a prevalent form of reproduction

Sexual reproduction is the predominant form of reproduction in eukaryotes and commonly understood as the basis for genetic diversity (Cavalier-Smith 2002). However, there exists in nature another form of reproduction known as *parthenogenesis* ("virgin birth"), in which an egg cell can develop into offspring without being activated by a sperm. In such cases, the offspring, as well as its siblings, are assumed to be genetically identical to the mother. Throughout the plant and animal kingdom, there exist asexually reproducing species, often besides close relatives, which keep reproducing sexually. Natural parthenogenesis was discovered by the Swiss naturalist Bonnet in 1745, who noted the phenomenon in spindle-tree aphids. Parthenogenetic reproduction especially occurs in plants, rotifers, nematodes and arthropods (Bell 1982), but also within vertebrates approximately 70 species have been identified to reproduce asexually, although none have been found in birds and mammals

(Avise *et al.* 1992, Lynch 1984). Nowadays, cladocerans, rotifers (Birky 2004, Gomez & Carvalho 2000, Welch & Meselson 2000), thelytokous earth mites, lake snails (Hughes 1989) and aphids (e.g. Haack *et al.* 2000, Simon *et al.* 1999, Sunnucks & Hales 1996, Sunnucks *et al.* 1997) are subject of scientific studies to learn more about the characteristics of asexual populations. Figure 1 illustrates two representatives of the plant and of the animal kingdom of actual research projects on asexual reproduction.

Modes of asexual reproduction

Parthenogenetic reproduction may occur via a wide variety of cytological mechanisms, retaining the meiotic division. Parthenogenetic reproduction, as described here, is based on thelytoky, the production of females from unfertilised eggs. Apomictic and automictic thelytoky need to be distiguished as the two main modes of asexual reproduction.

In apomictic parthenogens Mendelian segregation and crossing over do not take place and oocytes are produced by mitotic cell division in the germ line. Offspring hence inherits a complete unrecombined maternal genome, which results in increased levels of heterozygosity over time as mutations accumulate. Apomixis is found commonly in many aphid species (Blackman 1978, 1979, Hales *et al.* 2002, Sunnucks & Hales 1996, Wilson 2000) and is also supposed to be the mode of parthenogenetic reproduction in grape phylloxera, although nothing has been published upon this subject so far.

Automictic parthenogenesis in contrast to apomixis retains meiosis by conserving diploidy through duplication of the gametes produced by the parthenogenetic female. This usually rapidly leads to homozygosity, if not circumvented by endomeiosis, as practised by some species of lizards and grasshoppers (White 1973).

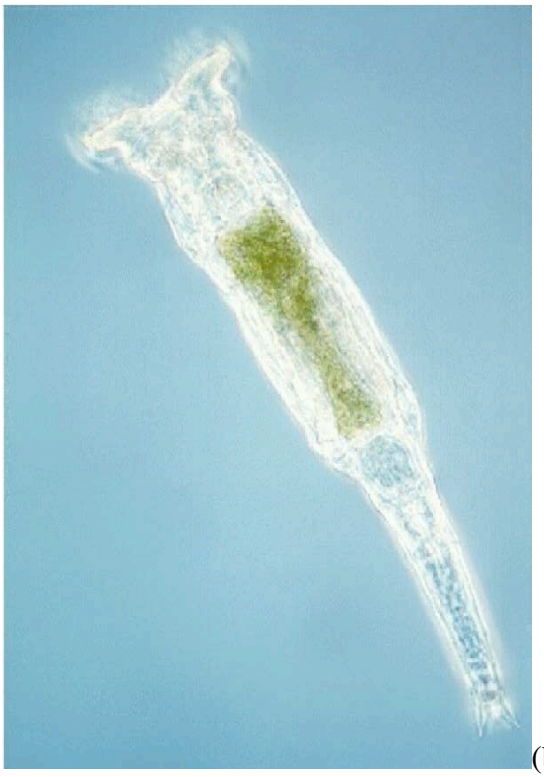
There are further mating systems closely related to parthenogenesis like gynogenesis (sperm from related species stimulates egg development) or hybridogenesis (half of the genome is transmitted sexually, half asexually), occuring for example in fish of the genus Poeciliopsis (see Beukeboom & Vrijenhoek (1998), Parker & Niklasson (1999), Simon *et al.* (2003), Vrijenhoek (1998) and White (1973) for further details).

Asexual versus sexual reproduction

Despite the wide variety of species reproducing asexually, sexual reproduction remains the predominant reproduction mode among multicellular organisms. Certainly, one is attempted to ask why there are different reproduction modes and why the most complex one, sexual reproduction, is so widespread - one of the most fundamental questions in evolutionary biology. Around this question, plenty of theories have been stated, explaining benefits and costs of both forms of reproduction.

Asexual reproducing populations can increase their numbers rapidly, since each individual is female and can produce eggs. In sexual populations, always two individuals, male

(a)



(b)

Figure 1: (a) Apomixis is the natural ability to reproduce asexually through seed (Nogler 1984) and is widespread among several plant families including Gramineae, Compositae and Rosaceae, but only in few crop species. The genus of *Taraxacum* is one of the model organisms to analyse the inheritance of the apomictic trait. By providing a system to produce seeds genetically identical to the mother plant, apomixis is a highly desired agricultural trait, but the mechanisms controlling this mode of reproduction are not yet completely defined (Van Dijk, 2003).
(b) Bdelloid rotifers are microscopic invertebrates, which have evolved for at least 40 million years without sexual recombination and therefore are called "ancient" asexuals. Their great evolutionary success seems to contradict all population genetic theories claiming that asexuals have a shorter evolutionary life-span than sexual reproducing organisms. For this reason, bdelloid rotifers have become important model organisms to analyse evolutionary effects of asexuality. Despite their asexuality, bdelloid rotifers have diversified into more than 360 species and occupy freshwater habitats worldwide (Birky 2004, Welch & Meselson, 2000). (photo: *Philodina roseola*, ca. 400μm, D.M. Welch, 2004).

and female, are needed to produce offspring, meaning that an asexual population will have the double rate of population increase under „all-else-is-equal“ conditions compared to the sexual one, which is called the „two-fold cost of sex“. (Maynard-Smith, 1978)

Sexual reproduction will only be worthwile, if the costs of this reproduction mode are compensated by other advantages. Most theories fall into one of two categories: (1) Sex increases the rate of adaptive evolution by generating new gene combinations (*Tangled Bank Model* or *Best-Man-Hypothesis*, described by Bell 1982, Ghiselin 1974 and Williams 1966) and (2) Sex prevents the accumulation of deleterious mutations (*Mullers ratchet*, described by Lynch *et al.* 1993, Müller 1964 and Kondrashov 1985).

In evolutionary time scales, sexual reproduction seems to offer a net advantage by allowing more rapid adaptation to changing environments. However, asexual reproduction may have short term benefits when rapid population growth is important or in stable environments. Asexual populations are excellent in quickly exploiting the available resources (e.g. in spring) and will also perform better in settling isolated habitats, as only a single individual is needed to start up a new population (Blackman 1981, 2000).

The natural coexistance of closely related asexual and sexual taxa provide the best opportunity to analyse the advantages and disadvantages of both reproduction systems. Several model systems have been established on this purpose by evolution biologists: fish (Vrijenhoek 1998), snails (Lively 1987), cladocera (Hebert *et al.* 1989) and, of course, aphids (Halkett *et al.* 2006, Rispe & Pierre 1998).

Reproductive mode of aphids

There is probably no better way to learn about sexual and parthenogenetic reproduction than to look at the family of the Aphididae, which have adapted both systems to their benefit.

The life cycles within a single aphid species may cover various combinations of sexual and asexual reproduction. The typical annual cycle includes several parthenogenetic generations, cut by a single sexual generation, e.g. for overwintering (Moran 1992). Besides cyclical parthenogenesis, related populations of the same species may also reproduce by obligate parthenogenesis or rarely by parthenogenesis including male production (Dedryver *et al.* 1998).

Within many aphid species single populations or lineages have abandoned sexual reproduction: *Sitobion avenae* (Simon *et al.* 1999, Dedryver *et al.* 2001), *Acyrthosiphum pisum* (MacKay *et al.* 1993), *Rhopalosiphum padi* (Simon *et al.* 1991) or *Myzus persicae* (Vorburger *et al.* 2003), although their ability to reproduce sexually will usually not be lost. Many pest aphids tend to abandon their sexual phase in the greenhouse (Fuller *et al.* 1999).

Furthermore, aphid species being introduced to another continent or new habitat in general show tendency to abandon their sexual cycle. The spotted alfalfa aphid *Therioaphis trifolii* (forma maculata), for example, was introduced to North America in 1953/54 and it had already spread all over the Southern United States in 1956. In Europe and Asia, this

aphid is common, showing a variable morphology on several leguminous plants and sexual reproduction is predominant. In North America, only a single morphology on a single host, alfalfa, was detected and the aphid reproduced by obligate parthenogenesis. Lack of genetic recombination did not prevent the spotted alfalfa aphid from adapting quickly to its new environment. Resistances to organophosphorous insectides were identified already two years after the initial appearance of the insect in North America (Blackman 1981). The aphids then spread further to the Northern States, where it fell back to sexual reproduction to survive the colder climates in wintertime.

The ability to reproduce sexually may be lost spontaneously by mutation (Blackman & Brown 1991, Blackman & Eastop 1985) or by hybridizing with males, producing obligate parthenogens, which carry genes for asexuality (Blackman 1972, Simon *et al.* 2002, Simon *et al.* 2003). The latter mode may also be a hybridisation event between cyclical parthenogenetic populations with closely related asexual species (Delmotte *et al.* 2003, Vorburger *et al.* 2003). Another phenomenon, which makes parthenogenetic reproduction of aphids so powerful, is the telescoping of generations. Eggs within most aphids start to develop long before birth, so that a newly born aphid can contain within herself not only the developing embryos of her daughters but also those of her granddaughters, which are developing within her daughters. Dixon (1998) related this phenomenon to as "a biological Russian doll, one generation within the other". This contributes to rapid population growth and may also be a factor contributing to quick adaptation to changing environmental conditions.

Imperfect clonality

The offspring produced during clonal reproduction phases of aphids encompasses an immense number of putative genetically identical individuals. Producing large quantities of identical genotypes might be the initial aim of parthenogenetic aphid populations, but will nevertheless entail the aforementioned problem: the lack of genetic diversity.

Blackman (2000) ironically entitled his paper "The cloning experts" to illustrate this topic. He stated that aphids are not as good at "cloning" as they are expected. The genetic traits of a clone could not be graphically represented as "vertical poles", invariable within a Normal distribution with mean and variance (Loxdale & Lushai 2003). Imagining the genome size of a multicellular organism, e.g. an aphid, the authors assumed that this situation would be very unlikely. Mutations and other genomic changes may sneak into aphid's genomes and cause a certain level of genetic diversity (Lushai & Loxdale 2002). Furthermore, Dixon (1998) stated that mutations become a matter of certainty rather than chance when taking into account the immense number of individuals produced by parthenogenesis in aphid populations. By developing from a single cell stage, aphids even would have a greater chance of mutating compared to other asexuals reproducing e.g. by budding, as coelenterates. Lynch & Gabriel (1983) even claimed that the rate of phenotypic evolution might approach that achieved under obligate sexual reproduction.

But also factors other than mutation may influence the homogeneity or heterogeneity of a clone. Karyotypic variation associated with chromosomal rearrangements was shown to play an important role in host plant adaptation (Sunnucks *et al.* 1998, Wilson *et al.* 1999). Mitotic crossing over proved to be the reason for intraclonal differences on the X chromosome of the aphid *Megoura viciae* (Mandrioli *et al.* 1999). Moreover, simple differences in the number of ovarioles (and embryos) (Dixon 1998) point to unstable conditions in the reproductive system of so called clones.

Even if genomic changes within a clonal lineage remain unobserved phenotypically, these genetic changes can be easily analysed employing modern molecular biology marker tools. Using molecular markers with an appropriate resolution, more details of intraclonal genetic variability will be clarified: whether coding or non-coding regions may be involved or whether genetic changes may even have adaptive significance.

2.3 Clonal diversity

Molecular genetic tools for analysing clonal variation

Employing molecular markers helps to analyse the genetic structure and reproductive system of parthenogenetic organisms. Molecular markers have been employed for several years now to detect genetic variation among and within species (Loxdale & Lushai 1998, Sunnucks 2000). Even differences among individuals of clonal offspring, so called intraclonal genetic variation, can now be detected with fine scale genetic markers (Samadi *et al.* 1999, Simon *et al.* 2002, Sunnucks *et al.* 1997).

In order to perform significant studies of population genetic structure and intraclonal variation not only the choice of appropriate genetic markers is as important as the right sampling strategy and, furthermore, the careful statistical interpretation.

Sampling

Ideally, a sample should comprise a large quantity of individuals to portray the realistic situation as good as possible. But this is not always achievable (Constantine 2003). In order to obtain a realistic picture of the genetic structure present in a field population, all levels of spatial structure should be allocated. A hierarchical sampling scheme will then prevent hidden genetic structuring, such as the Wahlund effect (Maynard-Smith 1989, Ridley 2004) in the sample, which is essential for sampling in large populations, as in aphids. In addition to spatial hierarchies, also temporal aspects may be included in the sampling strategies (Sunnucks *et al.* 1997). When monitoring clonal populations, applying these sampling strategies carefully is very important, in order not to distort statistics, which will be very sensitive to the present mode of reproduction (Halkett *et al.* 2005).

Markers for analysis of intraclonal variation

The resultant genetic profile furthermore depends directly on the type of marker employed and its ability to resolve the differences among the samples. Studying inter- and intraclonal diversity, one has to keep in mind, that clonal genotypes could appear as perfectly identical when using a specific marker set and differ when using another one. Loxdale & Lushai (2003) therefore conspicuously denoted a clone as a function of marker resolution. To determine clonality, it is thus ambiguous to analyse a few loci and multilocus approaches seem to be preferable. Only direct sequencing of large parts, or even the entire genome, would reveal the definitive identity of individuals or clonal lineages (Caillaud *et al.* 2004).

Initially allozymes, polymorphic enzymes, were used as protein markers for population genetics or differentiation among aphid species (Blackman 1979, Loxdale *et al.* 1994). These markers were rather limited in number and resolution and were therefore replaced by various DNA markers involving PCR (polymerase chain reaction). PCR based markers are favourable when dealing with low quantities of biological material (e.g. analysing single aphid individuals), since very low amounts and even degraded DNA may be employed. Moreover, compared to allozymes, very specific regions of the genome may targeted (Sunnucks 2000).

Mitochondrial (mtDNA) markers were employed by Barette *et al.* (1994) and Shufran *et al.* (2000) using sequences of the cytochrome oxidase I coding region (COI) to discriminate between different biotypes of *Schizaphis graminum* (Rondani). These markers have further been used for phylogenetics in aphids and in relationship with their obligate endosymbionts *Buchnera aphidicola* (Buchner) (Funk *et al.* 2001, Thao & Baumann 2004).

Various single locus markers have been developed for detailed work on aphid population studies. De Barro (1994, 1995b) was the first to develop synthetic oligonucleotide probes from RAPD (restriction fragment length polymorphisms) markers in order to describe migration of single individuals and clonal lineages of *Sitobion avenae* (F.) in the field. Further non-coding genomic regions, like intergenic spacers (IGS) and rDNA internal transcribed spacers (ITS) were analysed for the geographic distribution of aphid clones (Fenton *et al.* 1998a, Fenton *et al.* 2003, Shufran *et al.* 1991, Shufran & Wilde 1994).

Finally, SSRs (simple sequence repeats), so called microsatellites, have conquered aphid laboratories in the past ten years. Microsatellites are made up out of small nucleotide sequences, which appear as mono-, di, tri- or tetranucleotide repeats in non-coding regions of every genome (e.g. A_9, CG_{11} or ATT_{10}). They are very sensible to mutations, highly polymorphic and are therefore considered an excellent tool for detailed population genetic studies on clonal lineages or of aphids. Population genetic studies using microsatellites have already been performed for a wide range of aphids (Figueroa *et al.* 2005, Llewellyn *et al.* 2004, Massonnet *et al.* 2004, Miller *et al.* 2003, Simon *et al.*, 1999, Simon *et al.* 2003, Sunnucks & Hales 1996, Vorburger *et al.* 2003). Microsatellites are very specific markers,

can hardly be employed over a wide taxonomic range and usually need to be developed for each species separately (Wilson *et al.* 2004). For *D. vitifoliae*, six microsatellites were developed by Corrie *et al.* (2002) and Vorwerk & Forneck (2006) and were employed for population genetic studies in this thesis (chapter 3). Seven additional microsatellites were recently developed and tested in California by Lin *et al.* (2006).

As described above using the example of aphids and grape phylloxera, microsatellite markers have "come into vogue" for assessing the reproductive mode of populations. However, caution is recommended when working with microsatellite markers, since they are highly polymorphic and very sensible to PCR conditions and it may frequently happen that three or more markers can be scored for a sample consisting of a single diploid organism.

Although microsatellites are employed as so called multilocus technique using several primer pairs in a single PCR reaction, they only cover few loci compared to AFLPs. AFLPs (amplified fragment length polymorphisms) were developed combining RFLPs (restriction fragment length polymorphisms) with PCR techniques (Vos *et al.* 1995). Variable primer sets generate high quantities of markers, displayed as so-called genetic fingerprints. AFLPs fall into the range of dominant markers, but have also proved useful in detecting polymorphisms in the phylloxera genome (Forneck *et al.* 2000, Hoy 1994, Lin *et al.* 1999, Vorwerk & Forneck 2007b).

Statistical demonstration of clonal reproduction

Halkett *et al.* (2005) proposed a simple and elaborated guideline for easy statistical analysis of clonally and partially clonally reproducing organisms. Clear signatures generated by the absence of genetic recombination can be worked up combining several statistical approaches, when codominant markers are used.

Since clonal reproduction prevents reshuffeling of alleles among the genome, one of the easiest signature detectable in asexually reproducing populations is the number of repeated multilocus genotypes, expressed in relation to the number of individuals sampled (G:N ratio) (Halkett *et al.* 2005). The presence of repeated multilocus genotypes cannot be taken as proof for asexual reproduction, since strongly subdivided populations tend to show the same result. Therefore, additional testing of linkage disequilibrium (LD) is recommended. Researchers working on asexual aphid populations have agreed on performing LD tests both with and without repeated identical genotypes in a sample. This is important, since sexual recombining populations or sudden bursts of single strains would also show significant linkage disequilibria – these can be distinguished from clonally reproducing populations by repeating the analysis without the repeated multilocus genotypes of the sample (Sunnucks *et al.* 1997). Finally, negative F_{IS} values, indicating excess in heterozygous individuals, give further evidence of clonal reproduction within a population (Balloux *et al.* 2003). A further test to gain more information on repeated multilocus genotypes present in a population can be performed by evaluating P_{sex} values (Stenberg *et al.* 2003). Threshold values

for P_{sex} can be estimated from Monte Carlo simulations using the program MLGSIM. Significant P_{sex}values indicate that multicopy genotypes are statistically overrepresented in a population and therefore a product of clonal amplification.

Extrachromosomal factors of intraclonal variation

When studying intraclonal variation, one has to keep in mind that genetic differences among clonal individuals may also be due to factors other than mutational events within the genome.

Recombination in parthenogenetic aphids may potentially be much more common because of a meiosis-like pairing and possible exchange of chromosomal material, called endomeiosis, which takes place during the development of parthenogenetic eggs. This process, proposed by Cognetti (1966), is controversial. Recent studies using highly variable multilocus DNA markers, did not detect endomeiosis in parthenogenetic aphids (Carvalho *et al.* 1991, Shufran & Wilde 1994, Simon *et al.* 1996b). Forneck *et al.* (1999), carrying out karyotypic studies on parthenogenetic ovaries neither observed any endomeiotic events in grape phylloxera.

Epigenetic effects, like methylation (or the absence of methylation, more effective in insects) of genomic regions may also play a role in intraclonal variation. Studies on insecticide resistance of *Myzus persicae* showed that the absence of methylated regions in structural esterase genes leads to differences in gene expression and therefore to differences in resistance to insecticides (Field *et al.* 2000) among otherwise identical individuals.

Moreover it is important to realize that, within an aphid, there is more than one genome present. Besides the chromosomes, mitochondria, obligatory and sometimes facultative symbiotic bacteria provide genetic material, when DNA is extracted from whole aphid bodies. Symbionts may be an important source of genetic variation within an aphid population (Abbot *et al.* 2002, Ferrari *et al.* 2004, Funk *et al.* 2001, Oliver *et al.* 2005). Most aphid species harbour obligatory symbiotic bacteria of the genus *Buchnera* (Buchner 1965). They live within specialized cells of the aphid, provide their host with essential nutrients and sometimes even control reproductive functions (Baumann *et al.* 1995, Charlat & Mercot 2001, Douglas 1998). *Buchnera* bacteria carry a reduced genome, are transmitted vertically via the ovaries to the offspring and survive only inside their host's body. The close association has even led to measurable signatures of co-evolution between aphid and its obligatory symbiont (Baumann *et al.* 1995).

Additionally, some aphids harbour facultative or secondary symbionts (Douglas *et al.* 2002), an association, which is not as close as the one with *Buchnera*. However, these symbionts, too, take over important functions and may therefore be essential for the fitness of aphid populations. Antibiotic functions were demonstrated for *Regiella insecticola* in the gut of *A. pisum* (Scarborough *et al.* 2005). Host specialisation may also depend on the presence or absence of symbiotic bacteria (Leonardo & Muiru 2003).

The presence of different genomes within an aphid needs to be considered carefully when

working on genetic variability of aphid populations, since these genomes may also be source of genetic variation.

The myth of the clone: empirical molecular evidence for rapid mutational change in asexual aphid lineages[*1]

The incidence of intraclonal variation in *D. vitifoliae* in this thesis was to be compared to another agricultural important aphid species, *Sitobion avenae* (F.). The grain aphid is one of the most important cereal pests in Europe (Blackman & Eastop 1985), feeding mainly on Poaceae, grasses and cereals (Hardy 1850). It displays various life cylce forms (holocyclic and anholocyclic) depending on climatic conditions (Delmotte *et al.* 2001, Llewellyn *et al.* 2003). Like grape phylloxera, the grain aphid does not alternate hosts.

Two single founder lineages of *S. avenae* were provided by the laboratory of Dr. Hugh Loxdale, Rothamsted, UK and analysed for intraclonal genetic differences employing AFLP markers. Eight clonal sublineages were selected each from the two *S. avenae* lineages, covering five generations and 156 individuals in total. For direct comparision, the same AFLP marker combinations as in the grape phylloxera study were applied.

S. avenae can be reared easily in in vitro systems. In this experiment, single founder lineages were reared in narrow isolation tubes containing wheat seedlings. Single individuals could be transferred to other tubes and thus, also traced easily throughout the experiment.

For the two *S. avenae* lineages analysed, 302 AFLP-markers were scored. 76 markers remained after exclusion of monomorphic markers. Applying AMOVA, variance components divided into 95.91% variance among lineages and 4.09% variance within lineages. Intraclonal variation was revealed by twelve polymorphic sites for the lineage DAV95 and 13 polymorphic sites for the lineage HF95a. In each sublineage, zero to six polymorphic sites were found and nucleotide diversity ranged from 0 to 0.029. As expected, when using AFLPs, more deletions than additional bands were registered in both lineages.

Even over this short term tested, the clonal lineages were not genetical identical and showed intraclonal variation. In seven sublineages, first mutations were revealed very early, in G_1 or G_2. In seven other sublineages, first mutations occurred in G_5. The latter may display statistical outliers, due to the very small sample size in early generations. Only two sublineages did not show any mutation.

As shown in grape phylloxera clonal lineages, most of the mutations seemed to be undirected, appearing and disappearing randomly throughout the generations sampled. Five markers, however, clearly revealed directed mutations and banding patterns changed from present to absent throughout several generations and also in several lineages.

[1]* The here presented data are based on analyses, conducted in cooperation with Dr. H. D. Loxdale, Rothamsted, UK, in order to compare intraclonal variation of grape phylloxera to another aphid species, *Sitobion avenae* (data unpublished).

This study confirms the idea that clonal lineages of aphids are not genetically identical populations. Using the multilocus AFLP technique, a large quantity of polymorphisms can be detected.

Yet, characteristics of the mutations detected in this study need to be further characterized, e.g. by sequencing. In contrast to grape phylloxera, *S. avenae*, possess obligate endosymbiotic bacteria, *Buchnera aphidicola*, which may influence the formation of mutations. The putative presence of foreign DNA sequences (endosymbiotic bacteria, viruses) is far more frequent in *S. avenae* than in *D. vitifoliae*. Furthermore, it would be interesting to know how clonal lineages of *S. avenae* performed in the field, considering the strong migration activity of this insect. Sunnucks *et al.* (1998) and Haack *et al.* (2000) already reported the presence of specialized, host-adapted genotypes for *S. avenae*. Together with a high reproductive rate, however, this may be another reason, why these parthenogenetic forms seem to be perfectly and rapidly adapted to new environmental conditions.

2.4 Habitats and Biotypes

Phylloxera habitats

Despite the introduction of grafted grapevines, grape phylloxera have not been completely banned from wine growing regions worldwide. The insect still survives in several habitats on susceptible plants, and seems furthermore highly adaptable to overcome the so-called resistances of American *Vitis* species. The decline of vineyards as result of this pest, and consequently losses of yield and quality of grapes, has been frequently reported in commercial vineyards (Granett *et al.* 1985, Boubals 1994). Moreover, galling reactions on leaves of European *Vitis* species, which are commonly not susceptible for the development of leaf galls, were reported by Boubals (1994), Kocsis *et al.* (2002), Presser *et al.* (1993).

European grape phylloxera inhabit three major types of habitats. A number of commercial vineyards are still planted with ungrafted material and here root-feeding stages of grape phylloxera encounter excellent conditions. Populations can also be found in rootstock nurseries where extensive pesticide treatments are required to produce pathogen-free plant material. Moreover, abandoned vineyards, in which rootstocks overtake the *V. vinifera* scions still represent an excellent habitat for grape phylloxera. Both rootstock nurseries and abandoned vineyards exhibit fully developed vines on which leaf and root-feeding stages can be observed.

Due to its monophagousity, habitats of grape phylloxera are restricted to wine growing regions. Infestations of viticultural sites or other *Vitis* habitats most likely occur through infested plant material or through cultivation techniques. Active migration is very limited due to the small size of the insect. This leads to isolated habitat patterns, in which each population forms its own genotypic pool. Analysis of the genotypic structure of natural grape phylloxera populations employing codominant microsatellite data revealed the absence

Figure 2: Rootstocks proliferate freely along the road (left side) close to commercial vineyards (Rouffach, France) representing an ideal habitat for grape phylloxera populations (photos Hartmann 2002, Dürr-Auster 2003) .

of overlapping genotypes among European habitats (Vorwerk & Forneck 2006, see chapter 3).

However, the insect seems to adapt quickly when it is introduced to a new habitat. Fast reproducing populations together with a high grade of genetic variability may be the perfect basis for the development of new biotypes.

Biotypes of grape phylloxera

Several definitions of biotypes have been proposed. Among these, we define a biotype as assigned to individuals being genetically identical sharing the same phenotypic trait or sharing a specific genotype. More generally, for agricultural pests, biotypes are often linked to specific types of environment or host plants (Diehl & Bush 1984, Granett *et al.* 1985).

Grape phylloxera biotypes are mainly based on characteristics of host utilisation. A first example of the development of new biotypes and the failure of phylloxera management by grafting was the widespread planting of AxR#1 in California in the 1960s. AxR#1 is a cross between one of the American species (*Vitis rupestris*) and the European grapevine variety, *Vitis vinifera*. While it seemed to be tolerant initially, its resistance collapsed when a new and more aggressive strain of phylloxera emerged in the vineyards (Granett *et al.* 1985). Granett distinguished between two biotypes: biotype A, which grew poorly on AxR#1 and biotype B, which did not harm other rootstocks but performed well on AxR#1.

The development of biotypes or strains specifically adapted to certain host varieties was evaluated in a number of laboratory assays, analysing the variability in aggressiveness of grape phylloxera on several rootstocks. Assays were mainly performed on exised root pieces (Kocsis *et al.* 2002, Granett *et al.* 1987) or aseptical dual cultures (Forneck *et al.* 2001). The work of Williams & Shambaugh (1988), Song & Granett (1990) and Forneck *et al.* (2001) showed that phylloxera populations perform better on their original host than on alternative hosts, where their fitness becomes restricted. Furthermore, it is suggested, that the host plant

of origin may pre-adapt phylloxera populations to utilize other hosts successfully. The development of simple isolation chambers (Forneck *et al.* 2001) allowed testing the interaction of single phylloxera clones with their host under realistic "whole-plant" conditions. The experiments performed by Ritter (2005) confirmed the postulations made on adaptational processes of this insect (chapter 5). Other aphid clones, e.g. of *Acyrtosiphon pisum* (Harris) or *Myzus persicae* (Sulzer), also revealed adaptational traits, related to the type host plant they were collected from (Fenton *et al.* 1998b, Via 1991).

A first genetic evidence for the existence of host-associated clones of grape phylloxera was given by Corrie *et al.* (2003). Using microsatellite markers and mitochondrial COI sequences, host-specific differences in population fitness parameters were revealed among the genotypic classes tested from field populations. So far, no further genetic evidence has been found for the existence of host related genotypes. In order to identify host related markers for a specific biotype, the adaptational process needs to be emulated under near-natural conditions, as attempted by Forneck *et al.* (2001) and Ritter *et al.* (2007, chapter 5). Genetic fingerprints, like AFLPs are useful to gain information on clonal lineages being transferred from their original host to a new host. Specific markers detected in fingerprints of well-performing lineages in such an assay, may be related to adaptational processes and could be tested as single locus markers in other lineages.

Parthenogenetic biotypes: generalists or specialists?

There have been numerous attempts to find out how clonal organisms adapt to a specific environment. In order to fit to their special niche and often to coexist with their sexual relatives, it was frequently argued, whether clonal organisms are rather generalists or specialists. Two main models were developed to characterize the association between asexual organisms and their environment: the "general purpose genotype" (Baker 1965) model and the "frozen niche variation" model (Vrijenhoek 1984). The "general purpose genotype" model was first developed in order to explain the broad geographical range of some parthenogenetic animals. Asexual populations of this kind are able to tolerate a wide range of environmental conditions and the high grade of heterozygosity lets them easily adapt to changing conditions.

For aphids the existence of so-called superclones occurring in a broad environmental and geographic range was already reported by several authors. For example two genotypes of *Myzus persicae* obligate parthenogenetic populations revealed to be very abundant and widespread across the Australian continent, representing up to 24% of the entire collection (Vorburger *et al.* 2003). Another two genotypes of asexual *Sitobion avenae* revealed to survive on host plants and to colonize large geographical zones of France over several years (Haack *et al.* 2000) The secret of this great ecological success of these genotypes and how they evolved still remains unexplained. Although researchers believe in the existence of superclones in grape phylloxera in other viticultural regions (e.g. in Australia: Dr. P. Sunnucks, personal communication, 2005) this seems unlikely for grape phylloxera populations in Eu-

rope. All habitats analysed so far, revealed a high level of genotypic diversity (Vorwerk & Forneck, 2006).

Alternatively one can suppose a clonal population to consist of highly specialized genotypes which are perfectly adapted to the habitat they live in. The “frozen niche variation” model suggests that clonal organisms may “freeze” the genetic variability of their sexual progenitors and then occupy niches they are perfectly adapted to. Both models show ways to utilize a high level of genetic diversity which is essential for adaptation. Whether parthenogenetic grape phylloxera can be assigned to one of these types will be discussed further in this thesis.

3 Reproductive mode of grape phylloxera (*Daktulosphaira vitifoliae*, Homoptera: Phylloxeridae) in Europe: molecular evidence for predominantly asexual populations and a lack of gene flow between them.[2]

Abstract

The genetic structure of European grape phylloxera populations, *Daktulosphaira vitifoliae* (Homoptera: Phylloxeridae) was analyzed using six polymorphic microsatellite markers. Genetic diversity data of six populations originating from Northern and Southern European viticultural regions was assessed for geographical differences and the structure of two additional populations was examined in more detail, and focusing on specific host plant and habitat characteristics. To test for 'signatures' of clonal reproduction, different population genetic measures were applied to the data obtained from these populations. A total of 195 multilocus genotypes were detected in 360 individuals tested. Significant deviations from Hardy-Weinberg equilibrium, negative F_{IS} values (from -0.148 to -0.658 per population), and the presence of multicopy genotypes revealed that the current major reproductive mode at each of the locations tested is asexual. The high genotypic diversity detected within and among populations however, together with the occurrence of unique *D. vitifoliae* genotypes indicate sexual recombination events, probably prior to the multiple introductions into Europe. The absence of overlapping genotypes between the sampling sites suggests low migration rates among the populations studied and implies that the main mode of insect dispersal is via infested plant material carried by human agency. The specific features of European *D. vitifoliae* habitats are illustrated to discuss the role of habitat and life cycle on the genetic structure of this globally important pest aphid species.

Introduction

The life history of a species and its spatial distribution can profoundly influence the pattern of genetic structure that it maintains. Breeding system, migration rates, and the size of local populations determine the homogenizing forces of mixing and recombination and the differentiating power of selection and genetic drift leading to the characteristic genetic structure of a given species (Rhomberg *et al.* 1985). Within the last few decades, the genetic structure of pest aphid populations has received increasing attention as a result of the evolution of insecticide resistance and that of biotypes overcoming plant resistance, as well as the spread of plant viral diseases (e.g. Field & Foster 2002, Guillemaud *et al.* 2003, Simon

[2]Vorwerk, S. & Forneck, A. (2006) Reproductive mode of grape phylloxera (*Daktulosphaira vitifoliae*, Homoptera: Phylloxeridae) in Europe: molecular evidence for predominantly asexual populations and a lack of gene flow between them. *Genome*, **49**, 678-687.

et al. 1999). The development of highly variable PCR-based molecular markers, such as microsatellite markers, have greatly facilitated the genetic analysis compared to allozymes, which show very little variation in aphid species relative to DNA-based markers (Hales *et al.* 1997, Johnson *et al.* 2000).

Grape phylloxera, *Daktulosphaira vitifoliae* Fitch (Homoptera: Phylloxeridae), are gall-forming aphids native to North America which are obligate plant parasites of grape (*Vitis* spp. L.). The pest was inadvertently introduced into Europe in about 1860 and its feeding on the highly susceptible rootstocks of European grape (*Vitis vinifera* L.) led to the widespread destruction of vineyards. It was further spread through infested plant material via human agency to South Africa and Australia, making *D. vitifoliae* an important grapevine pest worldwide. The development of resistant grapevine rootstocks bred from North American species enabled the redevelopment of European viticulture, as *V. vinifera* cultivars were grafted onto resistant rootstocks. Native *D. vitifoliae* populations showed varying performance in adaptability to new host plants (Downie *et al.* 2001, Forneck *et al.* 2001, Hawthorne & Via 1994). Association of aphid genotypes with specific host plants has been demonstrated in Australian *D. vitifoliae* clones (Corrie *et al.* 2003) and on European clonal lineages in greenhouse studies (Ritter *et al.* 2007).

Three habitats are now commonly inhabited by *D. vitifoliae* in Europe: commercial vineyards on rootstocks (root-feeding), rootstock nurseries (leaf- and root feeding) and abandoned vineyards in which rootstocks overtake the *V. vinifera* scions (root- and leaf feeding), the latter two being the main source of *D. vitifoliae* infestations. Extensive populations of *D. vitifoliae* can be found in rootstock nurseries and require extensive pesticide treatments to produce pathogen-free plant material. The decline of vineyards as result of this pest, and resulting in yield and quality losses of grapes, has been frequently reported in commercial vineyards (e.g. Granett *et al.* 1985, Boubals 1994).

The dominant reproductive mode of *D. vitifoliae* in Europe is difficult to elucidate solely based upon life-history and ecological approaches, mainly because of the difficulties experienced in observing and sampling sexual morphs. Winged males and females can be observed towards autumn, but sexual forms have a lifespan of only a few days (Forneck *et al.* 2000), limiting the chances of successful sexual reproduction and gene flow over long distances. However, no empirical evidence for the occurrence of overwintering eggs has been provided within the last 50 years or so. Recent studies based on microsatellite analysis in Australian vineyards suggest absence or negligible importance of sexual reproduction (Corrie *et al.* 2002). Other life cycle variations for *D. vitifoliae* have been reported from native American habitats (Downie & Granett 1998), suggesting that the life cycle described in the literature is not fixed and that populations may adapt to specific habitat conditions. Viviparous Aphididae inhabiting cold climatic zones may switch from a parthenogenetic mode of reproduction to become sexual, i.e. oviparous, laying diapausing, cold-hardy eggs, which are essential for overwintering. The same species can show combinations of sexual and asexual reproduction

in moderate climatic zones (Dixon 1998, Simon *et al.* 2002, Sunnucks *et al.* 1997). The primitive forms of the Aphididae, Adelgidae and Phylloxeridae, are oviparous for both sexual and asexual generations and may overwinter as parthenogenetic stages, which thereby skip sexual reproduction (see Granett *et al.* 2001 for details of the lifecycle). Molecular markers have been used in several previous studies to elucidate the genetic structure of the reproductive mode of *D. vitifoliae*. Genetic variation of this insect was initially studied by Fong *et al.* (1995) using RAPD markers. Further characterization of populations and definition of biotypes were conducted with AFLPs and at least two independent origins were suggested for the introduction of *D. vitifoliae* to European wine growing regions (Forneck *et al.* 2000). Phylogenetic analysis of mtDNA sequences showed that these two gene pools representing divergent *D. vitifoliae* lineages were introduced into global viticulture (Downie 2002) and are found in New Zealand and Australia.

The development of microsatellite markers for *D. vitifoliae* (Corrie *et al.* 2002) greatly facilitated assessment of reproductive mode of this insect. These molecular markers are especially suitable since they are predominantly selectively neutral (but see Li *et al.* 2002), co-dominant, single-locus markers, which often show many alleles per locus. Due to their co-dominance, single-locus microsatellites offer potential for tracking recombination events and relationships between genotypes (Goldstein & Schlötterer 1999). This is especially so for species with undefined or mixed reproductive modes, for which microsatellites have proved excellent research tools (Delmotte *et al.* 2002). In addition, individual multiple locus genotypes representing particular asexual lineages can be followed in time and space (Haack *et al.* 2000, Llewellyn *et al.* 2003, Simon *et al.* 1999, Sunnucks *et al.* 1997, Papura *et al.* 2003, Wilson *et al.* 2004).

In the present study, we have analyzed the recombination structure of introduced *D. vitifoliae* populations deriving from Northern and Southern European wine growing regions. We therefore compared the genotypic variation at six microsatellite loci among the first generation of leaf galling populations, the generation emerging directly from sexually recombined winter eggs, if sexual recombination took place, or alternatively from overwintering asexual forms. Two selected populations were examined in more detail over a single growing season. The main questions of interest were (a) to ascertain the predominant reproductive mode of European *D. vitifoliae* populations; (b) to assess the genetic structure and geographical distribution of holocyclic (alternating sexual and asexual life cycles) and anholocyclic populations (continous parthenogenetic reproduction); and (c) to examine the impact of leaf gall dominated habitats for populations of *D. vitifoliae* in Europe.

Table 1: Geographic region, habitat and host information of the *D. vitifoliae* samples tested in the present study

Collection Site	Host plant	Date of Collection	Study
Bingen, Germany	T5C (*V. berlandieri* x *V. riparia*)	06/2002	Geographic differentiation
Kientzheim, Alsace, France	C3309 (*V. rupestris* x *V. riparia*)	06/2002	Geographic differentiation
Rouffach, Alsace, France	T5C (*V. berlandieri* x *V. riparia*)	06/2002	Geographic differentiation
Orange, France	Rootstocks, not identified	06/2002	Geographic differentiation
Trèbes, France	SO4 (*V. berlandieri* x *V. riparia*)	05/2002	Geographic differentiation
Barcelona, Spain	Rootstocks, not identified	05/2002	Geographic differentiation
Bingen, Germany	T5C (*V. berlandieri* x *V. riparia*)	06/2003 (BiI) 08/2003 (BiII) 10/2003 (BiIII)	Habitat differentiation
Gundelsheim, Germany	SO4 (*V. berlandieri* x *V. riparia*)	06/2003 (GhI) 08/2003 (GhII) 10/2003 (GhIII)	Habitat differentiation

Materials and Methods

Sampling

Population samples of parthenogenetic, first-generation leaf galling *Daktulosphaira vitifoliae* were collected in 2002 from abandoned vineyards from various rootstock hosts (Table 1) at six sites throughout major European viticultural regions, and covering three northern areas (Bingen, Kientzheim, Rouffach) and three far southern ones (Orange, Trèbes, Barcelona) (Fig. 3). These locations were characterized by green shoots of rootstocks climbing on native trees or hedges, exhibiting optimal ecological conditions for leaf gallers. No pest management had been performed at any of the sites. The sites were separated by distances ranging from 30 to 1300 km. *D. vitifoliae* populations usually spread in a circular fashion, forming focal spots of infestation of 10-30 m^2 at these locations.

A hierarchical sampling scheme for asexual or partially asexual organisms in their natural habitats is essential to obtain a realistic picture of the observed populations under statistical analysis. Population substructuring effects such as the Wahlund Effect (large populations comprising sub-populations show fewer homozygotes than the average for the set of subdivided populations (Maynard-Smith 1989, Ridley 2004) may have strong influence on linkage disequilibrium tests and F-statistics and can be avoided by appropriate sampling schemes.

Sampling was conducted employing this hierarchical scheme: three leaf-galled shoots (2 m long) were randomly chosen - one from the center and two from the periphery of each

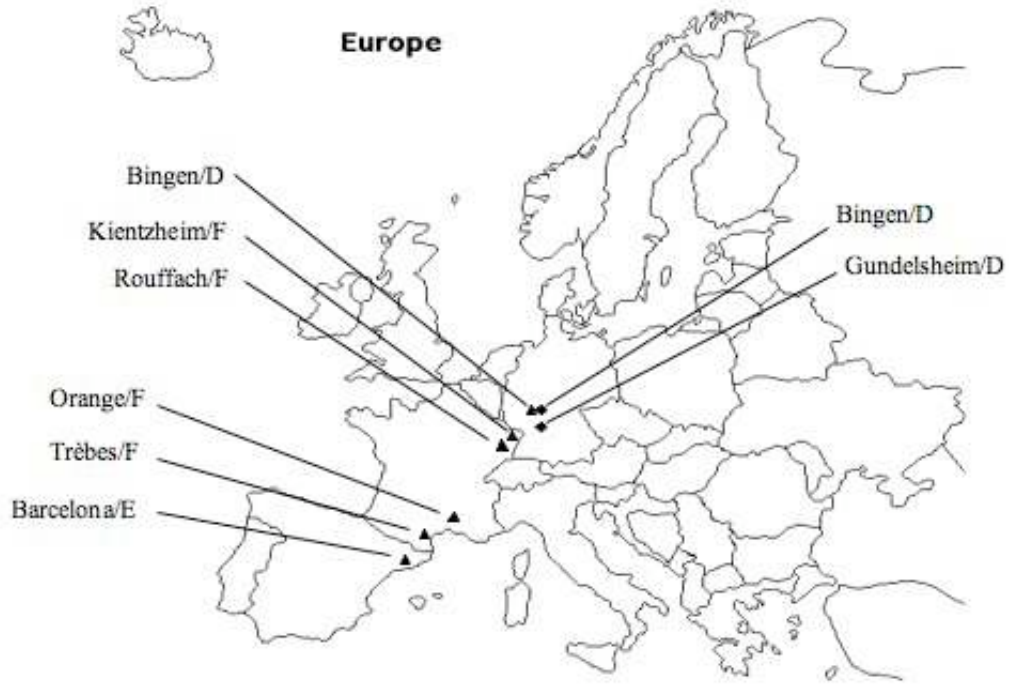


Figure 3: Map of collection sites for European *D. vitifoliae* populations (triangles = populations used for the spatial screening, diamonds = temporal screening)

location. Four to five gall-infested leaves were randomly chosen from each shoot and two to three single leaf-feeding adults were obtained by dissecting the galls and thereafter immediately frozen at -20 ˚C. In addition to these samples, a subset of two collections sites was sampled in 2003 to examine the temporal scale of genetic structuring of *D. vitifoliae* populations (Table 1): Bingen, which represents a spacious accumulation of hedge-like growing rootstocks with intermingling shoots where aphids can disperse freely along and between shoots; and Gundelsheim, a rootstock nursery, with individually pruned vines, which limits its population size and spread. Samples were taken three times during the season starting with the first leaf galling generation and consisted of thirty adult leaf-feeding phylloxera collected according to the scheme described above. Considerable care was taken to consistently sample the very same shoot of each plant.

Collection times were chosen to sample the first generation of parthenogenetic females. It was not known whether these generations arose from sexual recombined diapausing eggs or from hibernating parthenogenetic individuals. No phenotypic markers exist to indicate whether an individual originates from previous clonal or sexual reproduction. However, the populations tested were generally able to develop sexual stages, as shown under controlled greenhouse conditions in previous studies (Forneck *et al.* 2001).

DNA extraction and amplification of microsatellite loci

Total genomic DNA was extracted from single winged adult *D. vitifoliae* using a column-based DNA extraction kit (QIAGEN, Hilden, Germany). PCR reactions were performed in

Table 2: Microsatellite loci and primer combinations

Primer Name	Sequence 5'-> 3'	Repeat	Author	Annealing temp.	Allele sizes
Dvit1	5'CGTTCGTTCTGGTATCG TTATT3' 5'TAACGACCCGACTGAA ATGTAG3'	$(CA)_9(CG)_{14}$	Corrie *et al.* 2003	50 řC	128-142
Dvit2	5'GCTTAATTTTGTGTCTCA AGTTA3' 5'TAATGCTTCGTTTTCT AAGTGC3'	$(CT)_{14}(AT)_{16}$	Corrie *et al.* 2003	47˚C	257-289
Dvit3	5'CCAAAACAACCAAGATT TTCTCC3' 5'GATCCAAACTATGACAA ACACCC3'	$(AT)_9(GT)_{11}$	Corrie *et al.* 2003	50˚C	171-190
Dvit4	5'TCTTCAAAAATGTTA CATGAT3' 5'TATACAATGAATGGTAT CAATTC3'	$(AAT)_{11}$	Corrie *et al.* 2003	49˚C	156-171
Dvit5	Unpublished	$(A)_{13}$	Viduka, unpublished	Touch down 55-45˚C	126-142
Dvit6	5'TGGACGATGGTTTTC ATAGC3' 5'TTGATTGTCATTGG TTTTGC3'	$(AAT)_9$	Vorwerk & Forneck 2006	Touch down 57-47˚C	199-211

10 μl volumes containing 10 pM of each primer, 2 mM dNTPs (Fermentas, St. Leon Rot, Germany), 1 μl 10 x PCR Buffer, 1.5 mM $MgCl_2$ and 0.2 U *Taq* Polymerase (Invitrogen, Karlsruhe, Germany) and approximately 5 ng of template DNA.

PCR primers were used to amplify loci *Dvit1*, *Dvit2*, *Dvit3* and *Dvit4* (as described by Corrie *et al.* 2002), and *Dvit5*, an $(A)_{13}$ repeat (unpublished, primer sequences supplied by K. Viduka, La Trobe University, Australia) and *Dvit6*, an $(AAT)_9$ repeat flanked by the primers 3'-TGGACGATGGTTTTCATAGC-5' and 3'-TTGATTGTCATTGGTTTTGC-5' developed by our group (a sequenced allele can be found at the EMBL database: AJ969129). PCR conditions were as in table 2. Samples were electrophoresed on 5% polyacrylamide gels and silver stained as described in Forneck *et al.* (2000). A subset of every sample was double checked on an ALF - sequencer (Amersham Biosciences, Freiburg, Germany) employing 5'end cy5-labeled primers. Allele sizes were assigned using internal 200 bp and external 50-500 bp DNA size standards (Amersham Biosciences, Freiburg, Germany) and anchor samples of sequenced alleles originating from the study of Corrie *et al.* (2002).

Population genetic analysis

Genetic data for all six loci were combined and genotypes assigned to each sample. 17 genotypes bearing missing data were eliminated from calculations (Table 3). To prevent distortions of estimates for heterozygosity and F-statistics due to the presence of multiple copies of clonal genotypes in the populations tested, a single copy of each multilocus genotype was applied in the data analysis for F-statistics, estimates for heterozygosity, Hardy-Weinberg exact tests and linkage disequilibrium according to Sunnucks *et al.* (1997).

Allele frequencies, mean number of alleles per locus, observed heterozygosity (H_{obs}) and unbiased estimates of heterozygosity expected under HW assumptions (H_E) (Nei 1978) were processed using GENEPOP v. 3.4 online (http://wbiomed.curtin.edu.au/genepop/; Raymond & Rousset, 1995). This program was also employed to test data sets for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD). Exact P-values for these tests were calculated using the Markov chain method (Guo & Thompson 1992). FSTAT 2.9.3 (Goudet 1995) was employed to perform $F-$statistics on all population pairs, whilst confidence intervals based on bootstrap re-sampling were estimated according to Weir & Cockerham (1984).

Multicopy genotypes and population differentiation

An index for clonal diversity within populations was calculated for each population as k = G/N, where G is the number of different multilocus genotypes present in the sample and N is the sample size.

To gain more information on the multiple copy genotypes present in the populations and their likelihood to originate from clonal reproduction, the program MLGSIM developed by Stenberg *et al.* (2003) was employed. P_{sex} values were calculated for every multicopy genotype in each population as suggested by Halkett *et al.* (2005). Thresholds for P_{sex} values were estimated for each population from Monte Carlo simulations. Overrepresentation of multicopy genotypes result in significant P_{sex} values, giving further indication for clonal reproduction.

F_{ST} measures were transformed into F_{ST} / (1-F_{ST}) and a permuted correlation was computed against the natural logarithm (Ln) of distances between the sampling sites in kilometers, using the program ISOLDE (GENEPOP v. 3.4, http://wbiomed.curtin.edu.au/genepop/; Raymond & Rousset, 1995). The observed Spearman rank - correlation coefficient was compared under the null hypotheses that the two variables are independent.

The distribution of alleles and genotypes across all populations was tested using GENEPOP v. 3.4 unbiased tests of population pairwise differentiation (Fisher's exact test).

Results

High genotypic diversity and predominant clonal reproduction in European *D. vitifoliae* populations

Two to six alleles per locus were delineated, ranging in frequency from 0.017 to 1.000, and representing 107 genotypes for the 180 individuals sampled across the geographical locations. The statistical data analysis indicated predominantly clonal reproduction in European *D. vitifoliae* populations. Due to missing data, eleven genotypes were eliminated from the dataset of spatial differentiation and six from the dataset of temporal differentiation respectively.

Multicopy genotypes were found in all populations sampled. In the dataset of spatial differentiation, 80 genotypes were found to be unique - the remaining 89 individuals tested sharing a total of 27 genotypes. Based on the observed allele frequencies, the probability of independently produced repeated genotypes by sexual reproduction was calculated. Highly significant P_{sex}-values indicated that most of the genotypes scored repeatedly in the samples were generated by clonal reproduction. However the detected number of non significant P_{sex}-values bearing unique genotypes suggested that sexual recombination occurred, which may have prior to the introduction into European habitats (Table 3).

Hardy-Weinberg exact probability tests showed significant deviations from expectations at all collection sites and at all loci (Table 3). Mean observed heterozygosity ranged from 0.278 - 0.894 per locus. Negative F_{IS} values in all populations at all loci indicated an excess of heterozygotes with high variation among loci (Table 3). F_{IS} values ranged from -0.029 ($\pm$ 0.060) to -0.524 ($\pm$ 0.035) per locus and averaged -0.148 to -0.658 per population.

Predominant asexual reproduction irrespective of the geographic location

Due to the ecological link between colder climates and sexual reproduction reported for many aphid species (Delmotte *et al.* 2001; Simon *et al.* 2002; Llewellyn *et al.* 2003; Vorburger *et al.* 2003), it was hypothesized that Northern and Southern European *D. vitifoliae* populations differ in their reproductive modes. However, no significant differences were found between the two groups, both reproducing predominantly asexually. Both groups showed significant deviations from Hardy-Weinberg equilibrium and negative F_{IS} values, averaging -0.330 for the Northern populations and -0.382 for the Southern ones. The number of significant P_{sex} values differed among single populations (Table 3), without pattern with respect to geographic location. These differences may not be due to climatic factors, but also arise from specific population characteristics such as population size and migration rate within the habitat. For this reason, the P_{sex} parameter cannot stand alone as a measure used to detect clonal reproduction and needs to be seen in the specific population characteristics context.

Table 3: Population genetic parameters and datasets of geographic and habitat differentiation ($^{-MCG}$ indicates calculations without multicopy genotypes): number of individuals per sample, number of distinct genotypes, genotypic diversity index (G/N), number of repeated genotypes per sample and number of significantly clonal genotypes, mean number of alleles (no. alleles), mean observed heterozygosity (mean H_{obs}), mean expected heterozygosity (mean H_E), exact P values for Hardy Weinberg Equilibrium (P(HWE)), F_{IS} per locus and F_{IS} averaged over loci.

	Bingen	Kientzheim	Rouffach	Orange	Trèbes	Barcelona	BiI	BiII	BiIII	GhI	GhII	GhIII
Individuals	27	29	28	29	30	26	30	29	29	29	27	30
distinct genotypes	11	26	16	19	12	23	19	17	14	6	17	15
G/N	0.407	0.897	0.571	0.655	0.400	0.885	0.633	0.586	0.483	0.207	0.630	0.500
repeated genotypes	5	2	4	6	8	2	6	5	5	4	7	7
Sign. P_{sex}	4	2	4	5	3	0	5	5	4	3	1	3
no. alleles	2.167	3.667	3.000	3.167	2.667	2.833	2.833	3.000	2.500	2.000	2.833	2.500
Mean H_{obs}	0.241	0.701	0.417	0.414	0.328	0.589	0.483	0.379	0.374	0.143	0.389	0.361
Mean H_E	0.187	0.533	0.309	0.362	0.203	0.379	0.364	0.333	0.256	0.091	0.289	0.231
P (HWE)	***	***	***	***	***	***	***	***	***	***	***	***
F_{IS} per locus $^{-MCG}$												
Dvit1	-0.200	-0.140	-0.579	-0.216	-0.846	-0.680	-0.027	0.256	-0.389	-1.000	-0.606	-0.875
Dvit2	-0.333	-0.027	-0.048	0.195	-0.254	0.016	-0.070	-0.017	-0.529	-0.250	-0.304	-0.400
Dvit3	NA	-0.282	-0.304	-0.200	-0.048	-0.073	-0.810	-0.391	-0.625	-0.111	-0.231	-0.167
Dvit4	-0.538	-0.538	-0.579	-0.333	-0.692	-0.578	-0.583	-0.646	-0.444	-1.000	-0.413	-1.000
Dvit5	-0.273	-0.431	-0.310	-0.247	-1.000	-0.384	-0.237	-0.254	-0.300	-1.000	-0.412	-0.591
Dvit6	-0.250	-0.563	-0.304	-0.189	-0.846	-0.209	-0.524	-0.529	-0.604	-0.111	-0.312	-0.175
F_{IS} multilocus $^{-MCG}$	-0.304	-0.325	-0.361	-0.148	-0.658	-0.339	-0.341	-0.144	-0.486	-0.667	-0.361	-0.595

*P<0.05, **P<0.01, ***P<0.001 (highly significant)

Table 4: Population pairwise F_{ST} (left side) and distances in kilometers (km) between the sampling sites (right side). Genotypic and genic population differentiation was highly significant for all pairs of populations.

	Bingen	Kientzheim	Rouffach	Orange	Trèbes	Barcelona
Bingen		260 km	290 km	880 km	1120 km	1320 km
Kientzheim	0.108		30 km	620 km	860 km	1060 km
Rouffach	0.255	0.198		590 km	830 km	1030 km
Orange	0.256	0.131	0.167		250 km	440 km
Trèbes	0.071	0.136	0.192	0.225		300 km
Barcelona	0.166	0.179	0.255	0.236	0.141	

No shared multiple-locus genotypes between Australian and European populations

Comparisons between Australian and European *D. vitifoliae* genotypes are based on the four microsatellite loci *Dvit1 - Dvit4*, since no datasets based on all six loci have as yet been published for Australian genotypes. Considering this reduced dataset of loci *Dvit1 - Dvit4*, no corresponding genotypes could be identified between Europe and Australia. However 68% of the total of alleles (loci *Dvit1 - Dvit4*) were shared between genotypes from the two geographic regions.

Population differentiation in European populations

Considering the full dataset of six loci, all genotypes of the six populations tested for the spatial screening and the two populations tested for the temporal differentiation were specific to the particular population concerned. Clonal diversity k (G/N ratio) ranged from 0.400 to 0.897. The distribution of allele frequencies and genotypes differed significantly for all pairs of populations (Fisher's method, $p < 0.05$). Population pairwise F_{ST} values, indicating genetic variation among populations, ranged from 0.071 to 0.256. There was no significant isolation by distance between sampling locations (Spearman's = 0.322, $p = 0.686$).

Only 29% (considering loci *Dvit1 - Dvit6*) of alleles were shared among all six populations. The two closest sampling locations, Kientzheim and Rouffach, separated by only 30 kilometers, shared 71% of their alleles, but none of their genotypes. Distribution of allele frequencies differed significantly between all pairs of populations (Fisher's method, $p <0.05$).

Differing population genetic structures in European *D. vitifoliae* leaf galling habitats

The population structure and genotypic composition of the two main types of *D. vitifoliae* habitats in Europe was examined for the populations from Bingen and Gundelsheim. Both habitats displayed strong deviations from HWE and negative F_{IS} values (-0.311 $\pm$ 0.115 for Bingen and -0.497 $\pm$ 0.103 for Gundelsheim). The main reproductive mode in both habitats was asexual. Analyzing multiple copy genotypes of the first sampling date in spring, five of six multiple copy genotypes for Bingen and three of four multiple copy genotypes

for Gundelsheim showed significant P_{sex} values. However, the type of habitat (cultivated *vs.* abandoned, natural) had an essential impact on the genetic structure reflected in the population statistical measures obtained.

Higher genetic variation, indicated by 50 multiple locus genotypes, of which 16 were repeated, was identified for the Bingen screening (natural habitat), whilst only 31 genotypes (14 repeated) were found for the Gundelsheim screening (cultivated habitat). No genotypes could be followed over time in the natural habitat Bingen, whereas 5 of the 31 genotypes could be followed over time in the cultivated habitat. The most common genotype, G_1, was found at all three collection times, whilst the genotypes G_5, G_{18}, G_{19} and G_{20} were found at two collection times.

Discussion

No experimental data have previously been available on the reproductive mode of *D. vitifoliae* populations in Europe, which were established following several introductions after 1850. This study examined in total 360 individuals from seven populations across Northern and Southern Europe and revealed that the predominant mode of reproduction of the populations studied was parthenogenetic.

Several clues as to the clonal nature of the reproductive mode of *D. vitifoliae* were evident in the datasets obtained, including strong deviations from HWE and negative F_{IS} values, which have also been reported for obligate asexuals of other aphid species (Delmotte *et al.* 2002, Papura *et al.* 2003, Vorburger *et al.* 2003). Considering the high variation among F_{IS} values within populations and loci and the occurrence of unique genotypes, rare sexual recombination events may play a minor role in the populations sampled (DeMeeus & Balloux 2004). These recombination events may have been occurred in the native habitats of phylloxera prior to introduction or re-introduction or may occur rarely today. For example sporadic sexual recombination among few individuals within a population. However clonal reproduction seems predominant in all European population samples tested in this study, thereby confirming the assumption that parthenogenesis is the predominant mode of reproduction in introduced populations of this pest aphid (Downie 2002). In addition, the presence of several multicopy genotypes in all populations provides further evidence for clonal reproduction. The underlying reasons for asexuality in European *D. vitifoliae* populations still needs to be addressed. A genetically-based loss of ability of European phylloxera to produce sexuals can be excluded, since all populations examined in this and previous studies showed the ability to produce sexual forms under environmentally-controlled laboratory conditions (Forneck *et al.* 2001).

Life cycle variation (holo- *vs.* anholocyclic) can be correlated along a gradient from higher to lower latitude in many aphid species (Aphidinae) such as the grain aphid, *Sitobion avenae* (F.), the bird cherry-oat aphid, *Rhopalosiphum padi* (L.) and the peach-potato aphid,

Myzus persicae (Sulzer), following the assumption that severe winters select for cyclical parthenogens and against obligate parthenogens (Delmotte *et al.* 2002, Llewellyn *et al.* 2003, Loxdale *et al.* 1993, Martinez-Torres *et al.* 1996, Martinez-Torres *et al.* 1997; Simon *et al.* 1999, Vorburger *et al.* 2003). Since most aphids are viviparous during parthenogenetic generations, it is widely assumed that obligate parthenogenesis can only be found in mild winter climates (but see Fenton *et al.* 1998a, Fenton *et al.* 2005, Loxdale *et al.* 1993). Two characteristics may favour obligate parthenogenesis in *D. vitifoliae*. First, it is oviparous in sexual and parthenogenetic generations. It may be guessed that parthenogenetically-produced eggs survive cold winter conditions. However, the climate of European wine growing areas may be mild enough to enable parthenogenetic *D. vitifoliae* hibernating first instars to survive the winters there. The average winter temperatures measured in Geisenheim, Germany (50ř00'N) were 0.7 ˚C and in Sevilla, Spain (37˚24'N) 10.3˚C (values are means of the past 30 years; Vogt & Schruft, 2000). Secondly, *D. vitifoliae* populations overwinter on the roots of their host, since no secondary host exists and leaves of *Vitis* ssp. fall in winter. This favours overwintering of parthenogenetic individuals as long as no severe ground frost occurs. Parallels can be drawn to other aphid species, living on the root part of their host plant, like *Pemphigus betae* or *P. bursarius* (Moran 1991, Phillips *et al.* 2000). The greenbug aphids, *Schizaphis gramini* (Rondani) may also overwinter parthenogenetically in thick tussocks of grass in northern latitudes of North America (Loxdale *et al.* 1993, Shufran & Wilde 1994).

The present datasets provided several indications of parthenogenesis playing an important role in European *D. vitifoliae* populations. However, additional examination of *D. vitifoliae* collected on roots during autumn and winter would shed more light on the overwintering mode of this insect. In contrast to other aphid species, we did not have the opportunity to compare apparent cyclical parthenogenetic with obligate asexual *D. vitifoliae* populations.

Spatial genetic variation and genetic separation of European *D. vitifoliae* populations

Specific genotypes were identified for each collection site, but no overlapping of genotypes was found. Even the two closest located collection sites in Alsace, northern France (Kientzheim and Rouffach), lacked overlapping genotypic profiles. This non-overlapping of genotypes and the strongly negative F_{ST} values indicate that the habitats of abandoned vineyards effectively prevent migration of genotypes over long distances (30-1300 km) in contrast to the deduced long range flight behaviour of other aphid species studied, e.g. *Sitobion avenae* in Europe (Llewellyn *et al.* 2003, Llewelyn *et al.* 2004). Such apparent short range migration has been documented in other aphids, such as damson-hop aphid, *Phorodon humuli* (Schrank) (Loxdale *et al.* 1998), *Myzus persicae* (Wilson *et al.* 2003) and in some gall forming aphids, notably *Melaphis rhois* (Fitch) (Hebert *et al.* 1991, Massonnet & Weisser 2004, Wool & Hales 2004).

Reasons for *D. vitifoliae* genotypes being restricted to single locations may be manifold.

Firstly, *D. vitifoliae* populations do not alternate hosts, because perennial host plants sustain the phylloxerids all year round. The formation of host plant and habitat-adapted genotypes is likely in a situation of restricted migration and monophagy (Stoetzel 1985, King & Buchanan 1986, Via 1991). In addition, if sexual forms developed, dispersal would be limited due to the small size and non-active flying of these morphs, as already shown by Corrie and Hoffmann (2004), leading to genetically differentiated populations. Infestations of viticultural sites or other *Vitis* habitats most likely occur through infested plant material carried between sites by human agency or through cultivation techniques, leading to unique population genetic patterns on each location and built up through small habitat-like bottlenecks. Lastly, different introductions of *D. vitifoliae* may play a major role in population differentiation in Europe (Downie 2000, Forneck *et al.* 2000). AFLP screenings of European and native American *D. vitifoliae* populations showed the presence of at least two distinct introductions of *D. vitifoliae* into Europe, which could be located to Northern and Southern European wine regions respectively (Forneck *et al.* 2000). Subsequent cytochrome oxidase I (COI) mtDNA sequence analysis has revealed that, though several introductions of *D. vitioliae* to Europe have been demonstrated, all European, Australian and New Zealand populations as well as those from the US Pacific Coast region have originated from North-eastern USA (Downie 2002). According to that study, the global assemblage of introduced populations appears to be less diverse than native *D. vitifoliae* populations.

If the scenario proposed here holds, bottleneck effects have played an important role and the introduced genetic variation would have been be packaged into diverse clonal genotypes, which may then have been distributed in a pattern determined by human influenced dispersal and the distribution of suitable host plants. In order to examine the founder effects more closely, it would be necessary to analyse native and introduced *D. vitifoliae* populations in more detail using a wider range of microsatellite markers than presently used here.

European *D. vitifoliae* habitats

Bingen and Gundelsheim are representative of the two habitat types commonly inhabited by leaf galling populations of *D. vitifoliae* Europe, the main source of infestation for neighbouring cultivated vineyards. The closer examination at three sampling times during one season revealed genetic structure differences between these locations.

Populations from the rootstock nursery habitat at Gundelsheim showed restricted genotypic diversity. At the first sampling, only six different genotypes were detected in a sample of 30, which may point to very few overwintering genotypes. The lower number of detected genotypes in Gundelsheim compared with Bingen can be attributed to the specific habitat characteristics. Cultivation methods maintain a restricted source of aphids and pruning limits their migration from plant to plant. Moreover, host plants in the habitat consist of a single *Vitis* clone and do not provide any need for phylloxera adaptation or diversification due to host adaptation, as was shown for vineyards consisting of several vine types (Corrie

et al. 2003). In contrast, the natural habitat at Bingen provides nearly unlimited resources for population development. Due to the different genotypes detected at each sampling date, we suggest that migration within the habitat plays an important role. Furthermore, clonal turnover may be rapid when population size increases. Large populations may be hosted in such habitats and provide excellent resources for genetic diversification. Although sexual recombination events seem to be irrelevant, mutations and rapid selection events may maintain diversity within the habitat (Figueroa *et al.* 2005, Loxdale & Lushai 2003).

Differences between Australian and European datasets may be caused by the different types of population and habitat analysed in these studies. Both types of leaf-galling *D. vitifoliae* populations in Europe may pass through major bottlenecks every autumn when host plant leaves senesce. This result means that few individuals within an entire leaf-galling population at each site in any year survive to the next year and there may be many forces selecting for survival. In contrast, root-galling populations exhibit different population dynamics, since roots remain throughout the seasons and phylloxera fluctuation occurs only through the dispersal of winged, sexual individuals or asexual crawlers leaving the existing population, leading ultimately to a more constant genetic profile. For long-term population dynamic studies, it would be necessary to examine both leaf galling and overwintering root galling populations.

Leaf galling populations represent the most important source of infestation for commercial vineyards in Europe, which they neighbour. Commercial vineyards, which are in close proximity to highly infectious leaf galling populations develop leaf galls and build up root galling populations. The high level of diversity of *D. vitifoliae* genotypes, despite predominant clonal reproduction, reveals the high potential for new biotypes to be present in these habitats.

Conclusion

Predominant clonal reproduction of European *D. vitifoliae* populations was observed in this investigation involving relevant population genetic parameters and following the arguments propounded in previous population genetic studies (Halkett *et al.* 2005, Sunnucks *et al.* 1997). The data furthermore indicates that sexual recombination events within European *D. vitifoliae* populations cannot be excluded, though the sources of this meiotic diversity, which depends on the variables of habitat, population size, introduction events and overwintering conditions, remain to be further analyzed.

However, since its first introduction into Europe a hundred and fifty years ago, *D. vitifoliae* populations seem to have rapidly diversified within a wide range of European viticultural regions. This may be due to mutations that arise within separated populations in their specific habitats, to rare sexual recombination events, to numerous re-infestation events through infested plant material from the native habitat North America *or*, from introduced habitats

elsewhere that have occurred continuously ever since the first infestation in the mid 19^{th} century.

After the containment of the phylloxera pest by the introduction of resistant rootstocks - abandoned vineyards and rootstock nurseries have remained as important sources for continuous infestations. Due to their perennial growth, these habitats allow populations to exist for a very long time and offer the development of unique populations of genotypes, populations that are rarely perturbed by migrating competitors. The rapid building up of populations during the summer months encourage migration and expansion to neighboring commercial vineyards. Consequently, there is significant potential for adaptation behind the high genetic diversity monitored, allowing genotypes to quickly adapt to new environmental conditions or habitats when and where possible.

4 Analysis of intraclonal variation in clonal lineages of grape phylloxera (*Daktulosphaira vitifoliae* Fitch) using AFLP fingerprinting and DNA sequencing[3]

Abstract

Two AFLP fingerprinting methods were employed to estimate the potential of AFLP fingerprints for the detection of genetic diversity present within single founder lineages of grape phylloxera (*Daktulosphaira vitifoliae* Fitch). Eight clonal lineages, reared under controlled conditions in a greenhouse assay and reproducing asexually throughout a minimum of 15 generations were monitored and mutations scored as polymorphisms comparing the founder individual to individuals of following generations. Genetic variation was detected within all lineages, even from early generations on. Six to 15 polymorphic sites were detected within the lineages, making up 4.3% of the total amount of genetic variation. The presence of contaminating extra-genomic sequences, as e.g. viral material, bacteria or ingested chloroplast DNA was excluded as a source of intraclonal variation. Sequencing of selected polymorphic bands confirmed their origin in mostly non-coding regions. AFLP techniques revealed to be a powerful tool for the identification and characterization of genetic diversity within clonal lineages. This assay showed clearly the potential of clonal lineages to develop diversity without sexual reproduction and the need for careful monitoring of clonally reproducing aphid pest species.

Introduction

The asexual reproduction of higher organisms has become an important field of research in the past years (Gill *et al.* 1995). "Natural" clones are thought of as being 'genetically homogenous', comprising genetically identical individuals and thus displaying genetic fidelity. It seems improbable, that asexually reproducing species transfer exact replicas of the maternal genome to their offspring over longer periods. Genetic changes are likely to slip in, possibly because of genetic or epigenetic mutations, endosymbiotic activities or yet unknown mechanisms, but only a few of them have been characterised to date (Hoy 1994, Loxdale & Lushai 2003, Lushai & Loxdale 2002).

Aphids provide excellent examples for eukaryotic organisms, having adapted partially to clonal reproduction by producing apparently identical offspring in a short time and thereby securing the continuity of their populations by an alternation of seasonally-based asexual and sexual generations (Dixon 1998, Simon *et al.* 2002). However, exact genetic identity in asexually reproducing aphids is rarely – if ever – maintained for long, as demonstrated

[3]Vorwerk S. & Forneck, A. (2007b) Analysis of intraclonal variation in clonal lineages of grape phylloxera (*Daktulosphaira vitifoliae*, Fitch) using AFLP fingerprints and sequencing. *Genome*, **50**, 1-8.

in a series of previous molecular studies that provide evidence of genetic variation within apomictic aphid lineages (De Barro *et al*. 1994, Field &Blackman 2003, Forneck *et al*. 2001, Lushai *et al*. 1997, Lushai *et al*. 1998, Lushai & Loxdale 2002). Intraclonal variation in aphid species has been demonstrated using different marker systems, for example allozymes (Brookes & Loxdale 1987, Simon & Hebert 1995, Tomiuk & Wöhrmann 1980), single locus markers, e.g. oligonucleotide probes (De Barro *et al*. 1994, Di Pietro & Caillaud 1998) and RAPDs (Black *et al*. 1992, De Barro *et al*. 1995, Lushai *et al*. 1998). All these markers concentrate on single or few specific loci within the clonal genome. They are not suitable to detect the quantity of genetic variability present among clonal individuals. Therefore, markers offering high-resolution scales are needed, for example AFLP (amplified fragment length polymorphism) markers (Vos *et al*. 1995). By combining restriction endonuclease digests with PCR, AFLP markers generate high numbers of reproducible bands and are likely to be randomly distributed in the genome. Polymorphic banding patterns result from DNA sequence alterations, which include mutations abolishing or creating new restriction sites and insertions or deletions between two given restriction sites. When compared with other samples using the same primer pair, the presence or absence of specific bands is indicative of genetic polymorphisms. AFLP marker sequences are unknown, but they can be cloned and sequenced easily. These "genetic fingerprints" generated by AFLP markers have proved useful in assays of interclonal differentiation, but may also have high enough resolution to be employed for intraclonal variation studies. The first attempts to detect polymorphisms within parthenogenetic phylloxera lineages were made by Forneck *et al*. (2000) and Hoy (1994).

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Sternorrhyncha: Phylloxeridae) is suitable to examine the mechanisms of intraclonal genetic variation in a group of insects taxonomically closely related to the *Aphidinae*. Phylloxera form galls and feed on leaves and roots of *Vitis* species (Davidson & Nougaret 1921). Unlike members of the *Aphidinae*, no evidence for host alternation has been documented. Recent studies using microsatellite markers have revealed that grape phylloxera preferably reproduce asexually: even so, a high level of genetic diversity has been detected in the field populations tested (Vorwerk & Forneck 2006) and stresses the potential for new emerging 'biotypes' (Eastop 1973). For example, 'aggressive' strains infesting vines of commercial vineyards have consistently been reported in Europe and elsewhere in recent years (Boubals 1994, Song & Granett 1990).

In the present study, AFLP fingerprinting methods have been used in an attempt to uncover genetic diversity present within single founder lineages of grape phylloxera. Clonal lineages were monitored and mutations were scored as polymorphisms among the founder individual and individuals of following generations. Our aim was to estimate the potential of AFLP fingerprints to detect intraclonal variation. This technique provides a useful tool for the characterization and identification of mutations, inducing genomic diversity within and among clonal lineages.

Materials and Methods

Insect material

Leaf-galling populations of grape phylloxera were collected at three sites in Germany (table 5), with single adult females and their eggs being collected from leaf galls. Single founder lineages were established by inoculating host plants held in isolation cages with the eggs of single females (Forneck *et al*. 2001). The single asexual females were stored at -80˚C as founder individuals for later DNA analysis. The plant material was propagated from dormant 2-node cuttings of the leaf-susceptible cultivar Teleki 5C (*Vitis riparia* x *Vitis rupestris*), free of insects and any other possibly contaminating microorganisms. Defined light and temperature conditions were applied to keep the insect's reproduction mode clonal.

Clonal lineages examined in this experiment were genotyped using six microsatellite markers (Corrie *et al*. 2002, Vorwerk & Forneck 2006) following the protocols described previously (Vorwerk & Forneck 2006).

Sampling and monitoring of clonal lineages

Lineages were surveyed for population development twice weekly. Generation times differed among lineages and sampling intervals were adapted for sampling single adult females of equal life stages. Samples consisted of two to ten single adult females for each generation (depending on the number of developed leaf galls). In order to prevent overcrowding effects and to provide equal host conditions, surviving insects were regularly transferred to new host plants, by cutting two phylloxera-infested shoots and transferring them to an uninfected, isolated host plant.

DNA extraction and AFLP fingerprinting

For AFLP fingerprints, founder individuals were compared with individuals from early (G1-G4) and later (G10-G15) clonal generations (the choice of the exact generation sampled was determined by population development and fitness). Samples from generations in between were not analysed in this experiment in order to keep a manageable sample size, but were kept for eventual further analysis.

DNA samples were prepared using a column-based extraction kit (QIAGEN, Hilden, Germany) to produce DNA of consistent quality and consequently, highly reproducible PCR products. AFLP fingerprints were generated using restriction enzymes *EcoRI* and *MseI* as previously described (Forneck *et al*. 2000). After a pre-screening, the primer combinations E(10)*/M(8), E(13)*/M(8), E(14)*/M(8), E(16)*/M(8), E(21)*/M(3) and E(21)*/M(17) were chosen (table 6). PCR products in the range between 100 and 350 bp generated the selective amplification were electrophoresed on 5% polyacrylamide gels on an automated laser fluorescence sequencer (1500 V, 34 W, 60 mA, 55˚C). A 50 bp DNA ladder (Amersham-

Table 5: Detailed sampling scheme for the eight clonal lineages analysed

Lineage	Original host plant	Collection site and date of inoculation	Sampling date of early generations and number of sampled individuals (in parentheses)	Sampling date of late generations and number of sampled individuals (in parentheses)
A	T5C (*V. berlandieri* x *V. riparia*)	Geilweilerhof, Ger (25. June 2003)	18. July 2003 (3) 31. July 2003 (8)	21. Jan. 2004 (5) 20. Feb. 2004 (4)
B	T5C (*V. berlandieri* x *V. riparia*)	Geilweilerhof, Ger (25. June 2003)	31. Aug. 2003 (1) 12. Sept. 2003 (10)	20. Feb. 2004 (4) 07. Apr. 2004 (6)
C	C3309 (*V. rupestris* x *V. riparia*)	Weinsberg, Ger (19. June 2003)	18. July 2003 (4) 18. Aug. 2003 (6)	07. Apr. 2004 (10)
D	C3309 (*V. rupestris* x *V. riparia*)	Weinsberg, Ger (19. June 2003)	09. July 2003 (4) 31. July 2003 (2)	06. Okt. 2003 (2)*
E	C3309 (*V. rupestris* x *V. riparia*)	Weinsberg, Ger (19. June 003)	09. July 2003 (3) 18. July 2003 (8)	07. Apr. 2004 (8)
F	T5C (*V. berlandieri* x *V. riparia*)	Bingen, Ger (30. June 2003)	18. July 003 (8)	13. Nov. 1003 (10) 04. Dec. 2003 (2)
G	T5C (*V. berlandieri* x *V. riparia*)	Bingen, Ger (30. June 2003)	18. July 2003 (8) 18. Aug. 2003 (2)	21. Jan. 2004 (6) 20. Feb. 2004 (5)
H	T5C (*V. berlandieri* x *V. riparia*)	Bingen, Ger (30. June 2003)	31. Aug. 2003 (3) 12. Sept. 2003 (8)	20. Feb. 2004 (5) 07. Apr. 2004 (3)

* lineage D ceased in october 2003, therefore only a reduced number of samples could be taken

Table 6: AFLP primers and adapter sequences used for fingerprinting assays

Adapter/Primer	Sequence (5- 3)
*Eco*RI adapter	CTCGTAGACTGCGTACC
	AATTGGTACGCAGTCTAC
*Mse*I adapter	GACGATGAGTCCTGAG
	TACTCAGGACTCAT
*Not*I adapter	CTCGTAGACTGCGTA
	TACGCAGTCTAC
E(0)	GACTGCGTACCAATTC
E(10)**	GACTGCGTACCAATTCACA
E(14)**	GACTGCGTACCAATTCAGG
E(16)**	GACTGCGTACCAATTCATC
E(21)**	GACTGCGTACCAATTCCTA
M(0)	GATGAGTCCTGAGTAA
M(3)	GATGAGTCCTGAGTAAAGC
M(8)	GATGAGTCCTGAGTAAATG
M(17)	GATGAGTCCTGAGTAAAGT
N(0)**	GACTGCGTAATTGGCCGC

* *indicates primers labeled with cy5 for laser detection on the sequencer

Biosciences, Freiburg, Germany) was used as an external standard. A subset of samples was run twice for each primer combination to determine reproducible peaks and select markers for data analysis.

Statistical analysis of intraclonal variation

Only reproducible AFLP markers were scored and expressed in binary data format. After a thourough reproducibility screening, 141 of 271 markers remained for statistical analysis. Multivariate statistical methods were applied using NTSYS 2.0 (Exeter Software, Setauket, NY). A similarity matrix was calculated using the simple matching (SM)-coefficient (Sneath & Sokal 1973). Principle component analysis (PCA) was performed to reveal the genetic distances among the samples and determine similarities and groupings. Lastly, Arlequin 2.0 (Schneider *et al.* 2000) was used for analysis of molecular variance (AMOVA) and to estimate variance components for AFLP-haplotypes within and among clonal lineages (inter- and intraclonal variation).

Generating larger fragments for sequence analysis of intraclonal variation

A modified AFLP protocol was applied in order to generate larger fragments between 200 and 700 bp and polymorphic fragments were cloned and sequenced. The cutter *NotI* was used instead of the 4 bp cutter *MseI* and was combined with *EcoRI* for restriction of total DNA. *Eco*- and *Not*-adapter sequences (table 6) were ligated to the restricted fragments. The nonselective primers E(0) and N(0)* were employed for pre-amplification (Forneck *et*

al. 2000) and primers E(21)* and N(0)* were used for selective amplification of AFLP fragments. The thermocycler program consisted of ten cycles touch down PCR from 58˚C to 50˚C (annealing temperature), followed by 25 cycles at 50˚C. Amplification products were electrophoresed on highly resolving precast EL800 Spreadex-Gels (Elchrom Scientific, Cham, Switzerland) (300 mA 10 V/cm, 53˚C, 400 min) and visualised using ethidium bromide.

37 polymorphic fragments were recovered from the gels for cloning and sequencing by incubating excised fragments in 15 μl of elution buffer (50 mM NaCl 2.5 mM $MgCl_2$) at 65˚C for 45 min. The eluted fragments were directly used for cloning into pCR2.1 TOPO vectors (Invitrogen, Karlsruhe, Germany) and transformed into electro competent TOP 10F *E. coli* cells. At least five positive clones were selected through kanamycin resistance and blue-white selection and positive clones were confirmed via PCR. Sequencing reactions (at least two replications per sample) were performed by GATC-Biotech (Konstanz, Germany).

Sequence analysis

Sequences were evaluated using the software 4peaks (vers. 1.6, http://www. mekentosj.com/-4peaks), to eliminate vector sequences, record fragment length and detect restriction sites at the ends of each fragment. Processed sequences were checked against the general EMBL nucleotide database in order to define potentially coding sequences and EMBL specific nucleotide databases (http://www.ebi.ac.uk/blast2/nucleotide.html) to check for contaminating prokaryotic and plant sequences . In addition, sequences were checked for open reading frames, using the program ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Results

Genotyping

The eight clonal lineages analysed in this experiment were genotyped using six microsatellite loci. Several individuals per lineage were tested. Six genotypes were identified from the eight lineages. Genotypes from the same collection site differed by only one locus, whereas genotypes from different collection sites differed at four or five loci. There was no genotypic variation detectable within the lineages (table 7). For each of the three collection sites, clonal lineages with different genotypes were analysed with AFLP markers.

Intraclonal genetic variation

One hundred fourty-one reproducible AFLP markers ranging from 100 to 350 bp, of which 71 were polymorphic, were scored for variation using six selective primer combinations revealing intraclonal variation in all eight single founder lineages of *D. vitifoliae*. Six to 15 polymorphic AFLP-loci were identified per lineage. All eight lineages could be significantly

Table 7: Genotyping of clonal lineages at six microsatellite loci (*dvit1-dvit6*)

lineage	*dvit1**	*dvit2**	*dvit3**	*dvit4**	*dvit5**	*dvit6**
A	136140	259261	171190	164171	126128	199208
B	136140	259261	171190	164171	126128	199205
C	136138	257259	171190	164171	130134	205208
D	136138	257259	171190	164171	130134	205208
E	136138	257259	171190	159164	130134	205208
F	136140	257259	171190	159164	130134	199205
G	136140	257259	171190	159164	130134	199205
H	136140	259261	171190	159164	130134	199205

* genotyping according to Vorwerk & Forneck (2006)

Table 8: Analysis of molecular variance for all eight lineages (monomorphic markers excluded)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among lineages	7	3329.100	24.45632	95.67*
Within lineages	148	163.720	1.10622	4.33*
Total	155	349.821	25.56254	
Fixation index FST	0.95673			

*significantly different (alpha < 0.0001) (1023 permutations)

distinguished by statistic means. The mixing of lineages (i.e. lineage contamination) can likely be excluded as a source of genetic variation within the lineages tested. The distribution of genetic variation analysed using AMOVA was 95.7% among lineages (interclonal variation) and 4.3% within lineages (intraclonal variation) (table 8). Nucleotide diversity ranged from 0.010 ($\pm$ 0.007) to 0.026 ($\pm$ 0.016) (table 8).

Characteristics and distribution of mutations

Genetic variation was present in every lineage from early generations onward as indicated by altered fingerprints compared to the founder individual's AFLP fingerprint. In all eight lineages analysed, at least one individual of the first generation sampled contained one or more polymorphic sites. In the whole data set of 156 individuals 123 mutated individuals were detected, each carrying up to ten polymorphic sites (table 9). Most polymorphisms were characterized as "random" mutations, which were not detected in later generations. Five markers (E13M8/252, E14M8/86, E21M3/111, E13M8/214 and E13M8/167) revealed continuously mutated loci in five lineages. Three AFLP markers revealed to be polymorphic in more than one lineage (E10M8/130, E10M8/152, E13M8/100) (table 9).

No specific pattern of intraclonal AFLP-derived variation was observed to correlate with a particular microsatellite based genotype: clonal lineages originating from the same sampling site and carrying the same genotype did not reveal the same AFLP fingerprint over the generations sampled.

Table 9: Characteristics of mutations identified with AFLP markers in the eight clonal lineages A-H

Lineage	Number of individuals	Number of mutated individuals	Number of polymorphic markers	Gained loci (0→1)/ lost loci (1→0)	Number of mutation-specific loci	nucleotide diversity (Tajima 1983)
A	21	19	7	3/4	2	0.014 (± 0.009)
B	22	12	12	4/8	1	0.015 (± 0.095)
C	21	20	15	4/11	1	0.025 (± 0.015)
D	10	9	9	4/5	0	0.026 (± 0.016)
E	20	18	6	2/4	1	0.010 (± 0.007)
F	20	5	14	3/11	0	0.016 (± 0.010)
G	22	21	15	4/11	1	0.012 (± 0.008)
H	21	19	8	3/5	2	0.013 (± 0.009)

Sequence analysis of polymorphic fragments generated with *EcoRI* and *NotI*

In addition to the conventional AFLP assay, larger AFLP fragments were generated using the restriction enzymes *EcoRI* and *NotI*. The resulting fingerprints contained ten to 15 reproducible fragments, ranging from 200 to 700 bp. In this assay fifty-one polymorphic bands were detected throughout the eight clonal lineages of which 37 were cloned and sequenced. These fragments ranged from 187 to 433 bp and contained either two *NotI* or one *EcoRI* and one *NotI* restriction site (Table 10).

Five of the 37 sequenced fragments corresponded to 18S rDNA sequences of the brown citrus aphid *Toxoptera citricida* (GenBank accession no. AY216697). All sequences were deposited in GeneBank under the accession nos. AM17780 - AM117803 and AM411383 - AM411395 (Table 10). The remaining 32 sequences did not show significant similarity to known sequences. Sequence comparisons to bacterial and plant sequence databases were performed in order to detect possible contaminations causing polymorphic banding patterns. However, no similarities to either bacterial or plant-specific sequences or chlorophyll DNA were detected (Table 10) and the 32 sequences were determined to be noncoding, since analysis detected no open-reading frames and the search against protein databases did not result in any significant similarity.

Discussion

Detection of intraclonal variation

The application of a multilocus marker system in this study, combined with sequencing, facilitated detection of intraclonal variation and allowed further characterization of the mutations detected. The detection of mutated individuals depends on the marker system used (Fenton *et al.* 2005, Loxdale & Lushai 2003) and AFLPs as random, high-resolution markers

Table 10: Sequenced polymorphic fragments from AFLP analysis using restriction enzymes *EcoRI* and *NotI*

Fragment name	Accession no.*	Fragment size	AFLP restric-tions sites**	Similarity to known sequences of EMBL databases*	Fragment name	Accession no.*	Fragment size	AFLP restric-tions sites**	Similarity to known sequences of EMBL databases*
AFLP1	AM117780	220 bp	Not-Eco	non-coding	AFLP23	AM117799	264 bp	Eco-Not	non-coding
AFLP2	AM117781	303 bp	Not-Eco	99% *Toxoptera citricida* 18SrDNA, partial sequence	AFLP24	AM117800	256 bp	Not-Eco	non-coding
AFLP5b	AM117782	268 bp	Not-Not	non-coding	AFLP25	AM117801	300 bp	Not-Eco	non-coding
AFLP6b	AM117783	268 bp	Not-Not	non-coding	AFLP26	AM117802	196 bp	Eco-Not	non-coding
AFLP7b	AM117784	192 bp	Not-Not	non-coding	AFLP27	AM117803	190 bp	Not-Eco	non-coding
AFLP8	AM117785	229 bp	Eco-Not	non-coding	AFLP30a	AM411383	302 bp	Not-Eco	99% *Toxoptera citricida* 18SrDNA, partial sequence
AFLP9d	AM117786	285 bp	Eco-Not	non-coding	AFLP33a	AM411384	220 bp	Eco-Not	non-coding
AFLP10	AM117787	326 bp	Not-Not	99% *Toxoptera citricida* 18SrDNA, partial sequence	AFLP34c	AM411385	213 bp	Not-Eco	non-coding
AFLP11	AM117788	284 bp	Not-Not	non-coding	AFLP35b	AM411386	204 bp	Eco-Not	non-coding
AFLP13	AM117789	206 bp	Not-Eco	non-coding	AFLP37-1d	AM411387	302 bp	Not-Eco	99% *Toxoptera citricida* 18SrDNA, partial sequence
AFLP14	AM117790	293 bp	Eco-Not	non-coding	AFLP40a	AM411388	220 bp	Not-Eco	non-coding
AFLP15	AM117791	302 bp	Eco-Not	99% *Toxoptera citricida* 18SrDNA, partial sequence	AFLP41a	AM411389	213 bp	Not-Eco	non-coding
AFLP16	AM117792	32 bp	Eco-Eco	non-coding	AFLP42a	AM411390	206 bp	Not-Eco	non-coding
AFLP17	AM117793	188 bp	Eco-Not	non-coding	AFLP45a	AM411391	283 bp	Not-Not	non-coding
AFLP18	AM117794	162 bp	Eco-Not	non-coding	AFLP46a	AM411392	293 bp	Not-Eco	non-coding
AFLP19	AM117795	358 bp	Not-Not	non-coding	AFLP49a	AM411393	220 bp	Eco-Not	non-coding
AFLP20	AM117796	242 bp	Eco-Not	non-coding	AFLP50a	AM411394	211 bp	Not-Not	non-coding
AFLP21	AM117797	187 bp	Eco-Not	non-coding	AFLP51a	AM411395	206 bp	Not-Eco	non-coding
AFLP22	AM117798	433 bp	Not-Not	non-coding					

*http://www.ebi.ac.uk/embl; **restriction sites identified at the beginning and end of the sequenced fragment

seem appropriate. The presented data demonstrate that clonally reproducing *D. vitifoliae* in Europe (Vorwerk & Forneck 2006), are genetically variable, and hence dynamic in terms of their genomes and are thus suitable for the study of intraclonal variability.

Continuous generation of genetic variation

The intraclonal variation detected in this experiment differs from that of models, in which it is widely accepted that clonal organisms tend to accumulate deleterious mutations, that eventually lead to 'mutational meltdown' and extinction of the lineage (Lynch *et al.* 1993, Moran 1996, Wernegreen & Moran 1999), as demonstrated for example in parthenogenetic *Daphnia* (Cladocera: Crustaceae) lineages (Lynch & Blanchard 1998).

In *D. vitifoliae,* mutations seemed to be constantly generated at random loci over 15 generations of clonal offspring. Since only clearly reproducible markers were selected for data analysis, the remaining polymorphisms could definitely be assigned to mutations within the clonal lineages analysed. However, the experimental design does not allow testing of every filial daughter of a parthenogenetic female (*D. vitifoliae* lays up to 200 eggs per generation). During oogenesis each oocyte may mutate individually, leading to a slightly altered clonal offspring in each generation. The ability to test a small subset of individuals of each generation of a clonal lineage may cause that only a small fraction of the real genetic variation will be detected and, moreover, that the impression of randomly generated mutations will be even more intense. If one was able to test all individuals of a clonal lineage, the pattern of mutations generated from generation to generation might be clearer.

Furthermore, the assay does not permit the identification of deleterious mutations. Under experimental conditions, such as in a greenhouse, especially high rates of genetic variation may be registered, because external selective forces (inclement weather, predators, pathogens) are reduced in comparison with natural conditions (Harrington 1994, Loxdale & Lushai 2003). But still, negative influences due to mal adaptation or other unknown factors of an artificial environment cannot be completely eliminated. These factors need to be considered, when exploiting datasets for the assessment of putatively aggressive strains in clonally reproducing field populations of *D. vitifoliae*.

Only five mutated loci characterized in the various lineages, were transmitted through subsequent generations to the 15^{th} generation (the last generation analysed). Whether these polymorphisms were due to germ line mutations and whether these polymorphic loci affected the fitness of a clonal lineage remains unknown. These loci, however, may be an excellent basis for the development of specific single locus markers, which could be tested on further clonal populations of *D. vitifoliae* for the linkage to adaptational traits.

No non-phylloxeran templates in AFLP fingerprints

The possibility that non-phylloxeran DNA templates (deriving from associated bacteria or ingested plant material) generated the polymorphisms detected within the clonal lineages examined could be excluded in this experiment. Total DNA extracted from adult aphids may consist of three types of DNA: genomic, cytoplasmic and bacterial (Vorwerk & Forneck 2007a). By analysing the sequenced polymorphic fragments against general and specific DNA databases, the absence of sequences deriving from genomes other than grape phylloxera's was confirmed. This is an important aspect, which has so far been disregarded by others using multilocus markers, and needs to be considered in further analyses of intraclonal variation. Researchers working on aphid clonal lineages will soon be able to check polymorphic AFLP sequences against the fully sequenced genome of *Acyrthosiphon pisum* (Harris) (Caillaud *et al.* 2004, Sabater-Munoz *et al.* 2006), so that misinterpretations due to contaminating sequences will be significantly reduced.

The present study also reveals limitations in the application of AFLP markers. Mutations, affecting coding regions of the genome are unlikely to be detected with these markers, as shown in the presented sequencing assays. Nonetheless, specific single-locus markers linked to specific coding regions may be developed on the basis of polymorphic AFLP markers.

Further applications of AFLP markers to reveal intraclonal genetic variation

AFLPs provide a range of options to develop specific markers for the detection of intraclonal variation (Brugmans *et al.* 2003). Variable and also monomorphic fingerprint regions could serve as templates to develop specific single locus markers such as SCAR (sequence-characterized amplified region) markers. If markers applied to more than one lineage this would be of special interest in grapevine sciences to gain more information about host adaptation of *D. vitifoliae*, whereof still very little is known. A first experiment monitoring performance variables of *D. vitifoliae* single founder lineages adapting to new host plants used additional AFLP markers and identified three putatively host plant specific AFLP markers (unpublished data).

Monitoring of clonally reproducing insect pest populations could be facilitated and the development of new and putatively aggressive strains could be easily detected. Identifying specific markers derived from AFLP patterns may help to check *D. vitifoliae* infected sites for highly variable and therefore putatively aggressive lineages. Moreover, universally variable genomic regions could be identified, which could serve as polymorphic hotspot regions, even in more than one aphids species.

5 Adaptational potential of grape phylloxera (*Daktulosphaira vitifoliae*) clonal lineages measured by host performance and molecular markers.[4]

Abstract

The performance of each thirty grape phylloxera clonal lineages were examined on Teleki 5C (rootstock) and Cabernet Sauvignon (scion) over five generations using easily measurable performance data. In addition, AFLP molecular fingerprints were evaluated for a subset of six clonal lineages. Clonal lineages generally performed better on their host of origin, Teleki 5C, than on the new host, Cabernet Sauvignon. This was shown by assessing the number of surviving clonal lineages per generation, the number of reproducing adult individuals, the generation time and the number of ovarioles. Genetic fingerprints revealed high grades of *inter*- and *intra*clonal variability in all six lineages examined. Two presumable host plant specific markers were identified. Genetically and phenotypically highly variable grape phylloxera populations may have potential for short-term adaptation to new host plants and to develop new and aggressive grape phylloxera biotypes. This needs to be considered for further plant protection strategies in viticultural management.

Introduction

Over the last years, an increasing number of fallow vineyards are developing in many European viticultural areas. These habitat niches build up, when scions cease and the rootstocks shoots take over, providing a food source for leaf-feeding grape phylloxera (*Daktulosphaira vitifoliae* Fitch) enabling huge populations to develop. The new habitats expand rapidly and occur among economically vineyards allowing migration of phylloxera populations. Although *Vitis* v*inifera*-based hosts are described to be not susceptible to leaf-feeding phylloxera, galls can be readily observed on peripheral vines, close to infested rootstock habitats. Like many aphid species, phylloxera show variation in host preference and thus host adapted phylloxera biotypes exist.

Phylloxera feeds solely on plants of the genus *Vitis* (L.), which exhibits a great range of levels of resistances. All common wine and table grapes (*Vitis vinifera* ssp.) are described susceptible on roots and resistant on leaves, whereas many American *Vitis* species (e.g. *V. riparia, V. rupestris, V. berlandieri*), used for rootstock breeding are resistant on roots and susceptible on leaves. Hybrids among European and American *Vitis* species are increasingly planted due to their enhanced resistance against fungal diseases though mostly lacking phylloxera resistance on leaves.

[4]Ritter, A., Vorwerk, S., Blaich, R. & Forneck, A. (2007) Adaptational potential of grape phylloxera (*Daktulosphaira vitifoliae* Fitch) clonal lineages. *Mitteilungen Klosterneuburg,* **57**, 116-122.

Biotypes of phylloxera have been described to occur on rootstocks of *V. berlandieri* x *V. riparia* heritage. The resistance mechanisms of the widely planted rootstock AxR#1, with one *V. vinifera* parent, was overcome by "biotype B" phylloxera (Granett *et al.* 1985) and aggressive biotypes were registered to attack rootstocks common to European vineyards (Boubals 1994), demonstrating the sensitivity of plant resistance to changes in the pest population. Furthermore, variability in aggressiveness of grape phylloxera on rootstocks was noted in laboratory assays a number of times (e.g. Askani & Beiderbeck 1991; Grzegorczyk & Walker 1998; Kocsis *et al.* 2002). The work of Forneck *et al.* (2001a), Song and Granett (1990) has detailed the aphid's adaptive ability and suggested that the host plant of origin may pre-adapt phylloxera populations to utilize other hosts successfully.

Host plant suitability to an insect pest can be measured by the pest's performance (Zeng *et al.* 1993). By measuring the relative performance on the original versus the novel host, the adaptation of a population can be assessed. A successful biotype may evolve if genetic variation for traits exists within the assemblage of genotypes under selection (Hawthorne and Via 1994). Most European phylloxera populations are dominated by asexual reproduction (Vorwerk & Forneck 2006) exhibiting numerous clonal genotypes. Inter- and intraclonal polymorphisms are typical in aphids (Dixon 1987) and must be considered when studying the adaptation of an aphid population.

This study was conducted to assess the variation within one phylloxera population for biotype formation. The suitability of two host plants, representing the *Vinifera* and the rootstock host range, were assayed by measuring adaptation of leaf-feeding phylloxera being transferred to the roots of a novel host. This is a realistic approach, considering that hibernation of phylloxera occurs usually as parthenogenetic morph on roots, which suggests that successful root-feeding may represent a bottle neck for survival of leaf-feeding phylloxera. By measuring relative performance on original versus novel hosts and comparing genetic fingerprints of phylloxera clonal lineages the following questions were addressed: (1) Can *Vinifera* vs. rootstock adapted biotypes be identified within asexually reproducing grape phylloxera populations? (2) How diverse are phylloxera clonal lineages in terms of host-adaptation? (3) Can performance parameters be supported by a molecular screening using AFLP fingerprints? And (4) Can markers linked to performance and adaptation be applied to define a biotype?

Material and Methods

Phylloxera material

Single founder lineages of *D. vitifoliae* were initiated by single leaf-galling females, randomly collected from a population (Bingen, Germany) in May 2004 on a *V. berlandieri* x *V. riparia* Teleki 5C host. The same collection site has been used in several other grape phylloxera studies (Forneck *et al.* 1999, 2001a; Vorwerk & Forneck 2006).

Inoculation was effected by placing 25-30 eggs of a single female (G_0) near the roots of the new host plant. The founder individual (G_0) was stored in 0,5 ml tubes at -20˚C until DNA extraction. Eggs of the surviving aphids of each generation were transferred to new, uninfected host plants.

Plant material and isolation chamber system

The plant material used was propagated from dormant 2-node cuttings of the rootstock Teleki 5C (*V. berlandieri* x *V. riparia*) and of *V. vinifera* cv. Cabernet Sauvignon. The cuttings were watered for four hours, dipped in 1% indole-3-butyric acid for 10 sec and rooted over a four-week period in jiffy-pots. In the following we refer to the host plant treatments as T5C (Teleki 5C) and CS (Cabernet Sauvignon).

Phylloxera performance was observed in "Simple Isolation Chambers", a greenhouse-based system employed according to Forneck *et al.* (2001c) with the following modifications: the soil was a 1:1:8 mixture from sand, vermiculite and potting soil (peat moss; 160–260 mg/l nitrogen, 180–280 mg/l phosphate, 200–350 mg/l potassium oxide, 80–150 mg/l magnesium oxide, pH ($CaCl_2$): 5–6). One pre-rooted cutting was planted into each 600 ml of soil soaked with 200ml water. Isolation chambers were prepared two weeks prior to inoculation. No fertilizer was applied to eliminate interacting effects to the phylloxera.

Phylloxera Performance (Experimental Design)

Performance of each thirty single founder lineages on two treatments, T5C and CS, was observed over five generations (G_1–G_5). The following parameters were assessed in each generation: Surviving lineages (measured following each generation), numbers of fourth instars per lineage per generation (reproducing adults), generation time (the number of days from hatching to egg laying) and estimates of the produced offspring (eggs)**.** The plant was evaluated by counting the number of nodosities (root feeding sites) per plant. In each generation, 50 eggs were sampled from five randomly chosen individuals and transferred to new chambers. In order to test for re-occuring adaptational traits over succeeding generations, the surviving lineages of the CS-treatment were re-inoculated after four generations to their original host T5C. For DNA extraction, five randomly chosen adult individuals were collected in each generation and separately stored in 0,2 ml tubes at -20˚C until DNA extraction.

To gain more information on reproductive performance, ovariole counts were performed on five randomized adults. Therefore, single individuals were placed on a microscope slide with 15 μl acridine orange (2,5 mg/l) and a coverslip was carefully applied from cranial over the aphid's body, to release the reproductive organs including the intestinal tract into the buffer. The fluorescent spheroidal ends of the ovarioles were counted via microscope (Zeiss Axioplan, filter: Zeiss 450-490 nm, FT 510, LP 520).

Table 11: Primer sequences employed for AFLP analysis

Primer code	Sequence (5'-3')
M(8)	GAT GAG TCC TGA GTA AAT G
M(17)	GAT GAG TCC TGA GTA AAG T
E(10)**	GAC TGC GTA CCA ATT CAC A
E(14)**	GAC TGC GTA CCA ATT CAG G
E(16)**	GAC TGC GTA CCA ATT CAT C
E(21)**	GAC TGC GTA CCA ATT CCT A

* *indicates cy5-labeled primers

Statistical analysis

Performance parameters were statistically analysed with SPSS (version 10.0). GLM (general linear model) procedures, Analysis of Variance (ANOVA, univariate), with varying dependent variables were accomplished. Binary matrixes of the AFLP analyses were scored for present or absent bands to reveal information about mutated individuals and/or mutated lineages.

AFLP fingerprinting

AFLP-fingerprints were generated from six surviving lineages comprising the founder individual (G_0) as well as samples of the first (G_1) and the last generation (G_5). DNA samples were prepared using a column-based extraction kit (Qiagen) with modifications described by Vorwerk & Forneck (2007b). Four primer combinations using 3'-cy5'-labeled primers were used: E(10)*/M(8), E(14)*/M(8), E(16)*/M(8), E(21)*/M(17) (table 11). PCR products were electrophoresed on 5% polyacrylamide gels on an ALF sequencer (Amersham-Biosciences) (1500 V, 34 W, 60 mA, 55˚C). A 50 bp DNA ladder (Amersham-Biosciences) was used as an external standard. AFLP markers were scored for presence or absence and expressed in binary data.

Results

Performance and genetic data were analyzed to estimate adaptation and biotype formation of *D. vitifoliae*. An original host lineage, collected from T5C, was considered adapted to a new host (Cabernet Sauvignon) when its performance was not significantly weaker than comparable lineages on the original host.

Better performance on original host

Most of the lineages performed better on T5C than on CS, which was reflected by all performance parameters analyzed. The number of surviving adult individuals was significantly lower on CS than on T5C (table 12). The generation time was significantly longer for lin-

Table 12: Comparision of performance parameters for the host plants T5C and CS.

	surviving lineages		reproducing adults		number of ovarioles	
Generation/ Treatment	T5C	CS	T5C	CS	T5C	CS
G_1	30	30	30	20	12,8	**
G_2	14	9	14	11	8,7	**
G_3	10	8	10	7	13,0	8,1
G_4	9	7	9	%	12,6	8,9
G_5*	7	3	%	%	13,2	**

* surviving lineages were re-transferred to their original host after G_4
** due to low small sample sizes parameters could not be clearly measured in these generations

eages on CS (20.6d +/-1.8) compared to T5C (19.1d +/-1.3). Lineages on CS could only be transferred after the G_2, because lineages were very unstable in G_1. T5C-lineages and CS-lineages differed significantly among numbers of reproducing adults in all generations (T5C (mean G_{1-4} 38.8 +/-68.4); CS (mean G_{1-4} 10.5 +/-27.7). The number of ovarioles could only be compared directly in G_3 and G_4. The two treatments differed significantly: 13.0 (G_3) and 12.6 (G_4) ovarioles were counted for lineages on T5C, whereas only 8.1(G_3) and 8.6 (G_4) ovarioles were counted for lineages on CS (table 12).

Surviving lineages were transferred in G_5 to the original host T5C. Seven out of nine T5C lineages survived the re-transfer, whereas solely three out of seven CS-lineages survived. However single CS-lineages showed host adaptive traits: five CS-lineages measured up to the T5C-lineages over the course of the experiment in terms of generation time and numbers of reproducing adults (data not shown).

Genetic variation within and among single founder lineages

Six single founder lineages were fingerprinted, comprising the founder individual and individuals collected from G_1 and G_5. For each lineage, seven to twelve DNA-samples from single individuals were tested (table 13). AFLP markers were selected for reproducible and mutation-specific markers. 185 reproducible AFLP-markers were generated, ranging from 49 to 356 base pairs, of which 124 were polymorph among all samples tested. Three to seven polymorphic AFLP-loci were identified per lineage. Two host-plant related polymorphic markers were identified. Marker “158” appeared in each of the three lineages and was found specific for T5C lineages. Marker “209” appeared in all three examined lineages and was identified specific for CS. No monomorphic markers, specific for either T5C or CS were identified in this experiment.

Table 13: Descriptive analysis of AFLP-screening of the individual clonal lineages tested

	number of individuals tested, including G_0	number of usable loci*	number of polymorphic sites	polymorphic markers**
T5C-5	12	155	7	98, 101, 130, 132, **158**, 188, 321
T5C-16	7	154	6	112, 152, **158**, 321, 110, 165
T5C-21	9	152	4	130, 132, **158**, 208
CS-38	11	152	3	110, 118, **209**
CS-42	10	152	6	177, 74, 132, 143, 165, **209**
CS-43	9	151	6	69, 74, 132, 165, 177, **209**

*less than 5,00% missing data
**markers in bold type indicate host specifity

Discussion

In this experiment, grape phylloxera clonal lineages revealed host related adaptational traits, demonstrated through both performance and genetic variation. Differences in performance were shown in examined lineages differing in the number of surviving lineages, generation time, numbers of eggs per adult and average numbers of ovarioles. In addition, genetic variation was detected by AFLP fingerprints within and among phylloxera lineages from the first generation onwards. We conclude a significant interaction between aphid and host plant with respect with the aphid's performance could be confirmed.

Better performance on original host and detection of single adaptated CS-lineages

Most of the clonal lineages performed better on their hosts of origin (T5C) than they did on the alternate host as shown by every parameter analyzed. Apart from surviving individuals in G_1, which has been demonstrated to act as a strong selection force in previous experiments involving various bioassays (e.g. Granett *et al.* 1985; Hawthorne & Via 1994; Forneck *et al.* 2001b; Kocsis *et al.* 2002), the generation time increased significantly in most of the CS clonal lineages (T5C-lineages 19.1 d +/- 1.3 vs. CS-lineages 20.6 d (+/-1.8)).

Additionally, we repeatedly observed up to two third decreasing body sizes of fourth instars feeding on the new hosts CS from G_2 on (data not presented). Changes in body size in relation to host plant factors were also detected by Wool & Hales (1997) and Wilson *et al.* (2003). Moreover, phylloxera on CS also differed in colour (greenish compared to normal yellow), indicating a change of physiological factors.

Only two lineages were considered adapted to their new host plant CS: lineage 42 and 43. Though adaptation of these two lineages could not be traced in the "number of ovarioles", both revealed surpassing data of the parameters "number of reproducing adults" and "generation time". These two lineages show at least a certain short-term adaptation to their

new host, however, considering the rather long-term parameter "number of ovarioles", their further performance remains to be analysed.

Evaluation of parameters employed

The often used intrinsic rate of increase (Birch 1948) as a fitness parameter for performance did not proof suitable for the bioassay applied in the experiment. Instead, life cycle parameters were applied according to studies previously published on phylloxera performance (e.g. Kocsis *et al*. 2002). These parameters, as the number of surviving lineages, the number of reproducing adults and the generation time are straightforward to evaluate and experimental errors may be minimized. In our opinion these data allow comparing reviews among labs and bioassays.

Additionally we introduced the number of ovarioles as a new parameter. This may provide a physiological parameter exhibiting linkage to yet unknown parameters of adaptation. T5C-lineages showed significantly more ovarioles than the CS-lineages reflecting the host change as interacting environmental factor. We consider the ovariole system an interesting parameter for phylloxera performance, since it is related to telescoping of generations. With telescoping of generations, three generations develop in parallel, the embryo inside the adult's body bearing already predispositions of the following generation in its body before its own birth. This characteristic allows a quick reaction to environmental factors like changes in diet, host plant or overcrowding (Dixon 1988).

High inter- and intraclonal genetic variation

The genetic variation traced among the six lineages reflects the high genotypic diversity present in the "Bingen" population (Vorwerk & Forneck 2006; Vorwerk & Forneck 2007b), which was chosen to increase the likelihood of detecting different performance types as presented earlier (Forneck *et al*. 2001c) and for detecting "specific" fingerprints or markers for adaptation. In previous experiments, phylloxera populations were shown to mainly reproduce asexually in European abandoned vineyards (Vorwerk & Forneck 2006), though a significant genotypic variation exists, resulting from earlier holocycles and also due to the intermingling structure of vines, which allow first-instar morphs to spread easily.

Intraclonal variation, occurring among asexually reproducing individuals of a single founder lineage, was demonstrated in all lineages tested in our study. AFLP-based polymorphisms were previously found in phylloxera clonal lineages. Genetic variation traced by AFLP-markers originating from any source other than mutation is not likely but cannot completely be ruled out, though parallel experiments on clonal lineages of grape phylloxera showed that viral, bacterial or plant genomic DNA ingested by the insect could be excluded as source of genetic variation. It was further demonstrated that AFLP fingerprints mostly reveal genetic variation within non-coding regions, which may be a reason for not being able

to directly correlate genetic markers to phenotypic changes (Vorwerk & Forneck 2007b).

Host plant specific markers

Evidence was found in previous studies that mutation rates in grape phylloxera are high enough to produce heritable genetic variation over short time scales (Downie 2003). In our study, two AFLP-loci were identified to be linked to the host treatments. Host-plant specific markers have not been described elsewhere according to our knowledge and provide a first step in understanding the mechanisms that exist in host adaptation of asexually reproducing grape phylloxera. Furthers studies are underway to analyse the relationship of these markers to host adaptation, their distribution in natural populations, as well as their pattern occurring in the study with single founder lineages. Further complementation is necessary to confirm these markers are underway.

Evolution of European grape phylloxera adaptational traits over time

When phylloxera was originally introduced into Europe 150 years ago, own-rooted *Vinifera* vines rapidly declined due to high susceptibility against the new pest. In this experiment, however, a *Vinifera*-adapted biotype was not identified, nor in other assays testing phylloxera performance on rootstocks and *Vinifera* host plants. In all recent experiments, the populations or lineages tested showed to be well-adapted to rootstocks and showed superior performance on these hosts.

There must have consequently been a change in adaptation by European phylloxera in the past 150 years. After the introduction of grafted vines in Europe, phylloxera re-adapted to rootstocks feeding on their roots and leaves. Grape phylloxera populations were able to develop fitness providing advantages in conquering new hosts, which may have been persevered through strategies such as pre-adaptation, telescoping of generations and maternal effects. Other yet not studied mechanisms could include the interaction of microorganisms as shown recently for grape phylloxera and *Pantoea* species (Vorwerk & Forneck 2007a) or for galled *Vitis* roots and *Metarrhizium* (Huber *et al.* 2005).

It may be that the *vinifera*-adapted lineages of former times were consecutively outperformed by the phylloxera lineages inhabiting to more vigorous rootstock habitats (higher plant pathogen resistance). There is evidence for *vinifera* x rootstock adapted phylloxera lineages in California, termed “biotype B” (Granett *et al.* 1985), however these lineages seem to be untraceable in the field today.

Conclusion

Results of this experiment demonstrate, that within leaf-feeding phylloxera populations, rootstock-adapted individuals can be differentiated when tested on root feeding sites. Phylloxera populations, though reproducing asexually, are composed of multiple clonal lineages,

which seem to generate and preserve high genetic variation. Within the sampling range of 30 different lineages two can be considered adapted within the first five generations, confirming results presented in Vorwerk & Forneck (2007b) and clearly show that high genetic variation increases the chances of sourcing host adaptated lineages in laboratory assays.

Still, very little is known about the way in which selective forces operate in clonally structured populations (e.g. Di Pietro & Caillaud 1998). We emphasise the importance for estimating the inter- and intraclonal variation of phylloxera lineages. These measures will be of importance for the study of host-parasite interaction of various rootstocks. Populations which are highly variable both genetically and phenotypically may have a higher potential for short-term adaptation. For economic viticulture, growing 20-30 years monocultural plantings it is fundamental to know the source and evolutionary history of populations in order to predict future adaptive potential. We believe that combined performance and molecular marker studies are a basic tool to appreciate this potential and the urgency of precluding an new invasion.

6 *Pantoea agglomerans*, associated bacteria in grape phylloxera (*Daktulosphaira vitifoliae*, Fitch)[5]

Summary

Grape phylloxera lack intracellular symbionts, but the gallicole form seems to be associated with a single microbial species. 16S and 18S rDNA sequences were used for identification of symbiotic material. A single bacterial species, closely related to *P. agglomerans* was identified in adult parthenogenetic individuals, their eggs and leaf gall tissue of several populations. Bacteria revealed to be culturable on simple media. The type of association between these bacteria and grape phylloxera is undoubtely different from the well-studied symbiotic relationship of *Buchnera* and other aphid species, but may certainly impact on host-parasite interaction.

Introduction

Daktulosphaira vitifoliae (FITCH) belongs to the family of Phylloxeridae within the Aphidoidea. This monophageous insect produces galls by feeding on leaves and roots of many grape species (*Vitis* spp.). Contrarily to aphids of the Aphididae, *D. vitifoliae* does not feed mainly on the phloem, but rather uses its strong stylets to penetrate directly into the parenchymatic zone and imbibe the cellular content (Rilling & Radler 1960). Interestingly, transmission of viral material to the plant has not been reported. Previous histological studies made by the author confirmed the descriptions by other authors (e.g. Breider 1952, Ponsen 1997). The inner structures of grape phylloxera clearly differ from those of phloem feeding aphids. In previous studies, no mycetomes or mycetocytes, containing symbiotic microorganisms, were identified in grape phylloxera with staining and microscopic methods (Vorwerk *et al.* 2005), thereby confirming previous observations made by both Krassilstschik (1889) and Maillet (1952).

Most aphid taxa contain at least one or more bacterial taxa - primary symbionts that belong to gram-negative *Buchnera* species as well as other phylogenetically distinct groups termed „secondary symbionts“. Primary symbiotic bacteria occur in obligate association with their hosts in specialized cells called mycetocytes situated next to the gut lumen (Buchner 1965, McLean & Houk 1973). They are maternally transmitted by infection of either the early embryos in parthenogenetic aphids or the sexual egg in sexual females (Baumann 2005). *Buchnera* provides their hosts with essential amino acids that are not synthesised by the insect (Douglas 1998). Nearly all members of the Aphididae possess endosymbiotic *Buchnera* bacteria (Douglas 1998, Munson *et al.* 1991, Szklarzewicz 2000, Untermann & Baumann 1990). Moreover, some species contain eucaryotic, yeast-like symbionts instead

[5] Vorwerk, S., Martinez-Torres, D. & Forneck, A. (2007a) *Pantoea agglomerans* associated bacteria in grape phylloxera (*Daktulosphaira vitifoliae*, Fitch). *Agricultural and Forest Entomology*, **9**, 1-8.

of *Buchnera* (Fukatsu & Ishikawa 1992, 1996). Primary symbionts are highly specialized to their life in host tissues and can therefore not survive outside the body of their host nor be cultured *in vitro*.

In many aphid species there are lineages that, in addition to *Buchnera* carry other facultative, more or less loosely associated bacteria generally known as secondary endosymbionts. Most secondary symbionts are not maternally transmitted, but horizontally transfered. Currently, PCR based and sequencing methods, have allowed the identification of symbiotic bacteria (Harada *et al.* 1996, Sandström *et al.* 2001). Although these bacteria did not evolve so closely with their hosts, important effects on aphid biology have been demonstrated including effects on growth and reproduction (Chen *et al.* 2000), resistance to parasitoid wasps (Oliver *et al.* 2003), tolerance to heat stress (Montllor *et al.*, 2002), and on the range of usable host plants (Tsuchida *et al.* 2004) among others.

Search on symbionts of the grape phylloxera (*Daktulosphaira vitifoliae*, Fitch) was performed by different groups at the beginning of the 20^{th} century, resulting in controversial discussions. Grassi (1912) found "pseudo-vitellus-cells" that perhaps contained symbiotic organisms. A nutritive function was accorded to these cells. Krassiltschick (1889) and Schanderl (1949) described granulated structures in parts of the hindgut, thought to harbour symbionts. Other scientist contradicted these views (Breider 1952, Maillet 1957). Buchner (1965) excluded the existence of *Buchnera* type endosymbionts in *Daktulosphaira vitifoliae* in his work on symbiosis and no further research on symbionts infecting these insects has been done since then.

To definitely assess the presence of bacteria putatively associated with grape phylloxera, we have re-investigated the problem making use of the highly powerful molecular methodologies currently available. The confirmed presence of microbial DNA would affect genetic research in this insect pest, especially on intraclonal variation, new emerging biotypes and their potential for adaptation to new hosts. It is therefore essential to find out to what extent microbial genomes are involved in these processes. Moreover, the identification of phylloxera-associated microorganisms would be of great importance to clarify and define the ecological coherences of the phylloxera habitat, host-parasite interactions as well as for the design and development of new plant protection systems against this insect.

Materials and Methods

Biological material

Leaf gall samples containing parthenogenic grape phylloxera were collected from various locations in Europe and North America, including rootstocks 5C (*Vitis riparia* x *Vitis berlandieri*), C3309 (*V. ruspestris* x *V. riparia*) and 110R (*V. riparia* x *V. rupestris*). Grape phylloxera were collected from *Vitis arizonica* in their native american habitat. Two positive controls were chosen, bearing endosymbiotic *Buchnera* bacteria: *Aphis fabae* (SCOPOLI) maintained

in greenhouse culture on *Pisum sativum*, and *Myzus persicae* (SULZER) population maintained on *Vitis vinifera* cv. Bacchus.

Special care was taken not to carry over contaminations from the body surface of the insects. Grape phylloxera samples employed for DNA extraction and for culturing assays were washed for 5 min in 70% ethanol. The remaining ethanol was washed off with ddH_2O for DNA extraction and with phosphate buffer (20mM KH_2PO_4, 30mM Na_2HPO_4 $2H_2O$) for *in vitro* culturing assays.

DNA extraction

Total DNA of individuals was extracted according to a modified protocol of Lin & Walker (1996). Twenty to thirty individuals of each sample were washed with 400 μl of buffer 1 (10 mM Tris-HCl, pH 7.8, 60 mM NaCl, 5 % w/v sucrose and 10 mM EDTA) and homogenized in 160 μl of the same buffer. 200 μl of buffer 2 (300 mM Tris-HCl, pH 7.8, 1.25 % v/v SDS, 5 % sucrose and 10 mM EDTA) was added and gently mixed. The mixture was incubated at 65 ˚C for 30 min. For precipitation of cell components: 60 μl of buffer 3 (3 M K^+ acetate pH 4.8) were added and the mixture was kept at -20 řC for 15 min. The supernatant was collected after centrifugation for 10 min at 14000 rpm. Two volumes of EtOH (100 %) were added and after 15 min incubation on ice the mixture was centrifugated for 15 min to pellet the DNA. The DNA pellet was washed with EtOH (70 %), dried under vacuum for 10 min and left to resuspend in 50 μl $TE_{0.1}$ (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Samples were kept at 4 řC.

PCR and sequencing

Two general primers were employed in the primary screening for microbial DNA present in *D. vitifoliae*: *16seqF1* (5'-CCACTGGAACTGAGAYAC-3') and *16seqR1* (5'-AGGTGTAGCGGTGAAATG-3') both based on an alignment of different Enterobacteriaceae (Martinez-Torres *et al.* 2001). In order to detect possible yeast-like endosymbionts, the primers *F18SA1* (5'-AGCAGGCGCGCAAATTACCCAATC-3') and *F18SB1* (5'-CCTTGTTACGACTTTTACTTCCTC-3') (Fukatsu & Ishikawa 1996) were used. An additional primer pair was designed after first sequencing results, *pagF* (5'-CACTGGAAACGGTGGCTAAT-3') and *pagR* (5'-CGGCAGTCTCCTTTGAGTTC-3') for more specific amplification of *Pantoea agglomerans* 16S rDNA in further populations of *D. vitifoliae*.

PCR reaction mixtures comprised 1x PCR buffer, 0.1 mM dNTPs, 1 μM of each primer, 0.05 U/μl *Taq*-Polymerase (Invitrogen, Karlsruhe, Germany), 5-10 ng of template DNA and sterile ultra pure water in a total volume of 50 μl. The PCR program involved one cycle of 3 min 94 ˚C and 40 cycles of 30 s at 94 ˚C, 45 s at the annealing temperature (*16seqF1/R1* at 50˚C, *F18SA/SB1* at 52˚C and *pagF/R* at 49˚C) and 45 s at 72 ˚C. The final extension step was 5 min at 72 ˚C. PCR products were electrophoresed in 1.4 % agarose gels and

ethidium bromide stained. Amplified fragments were cloned using the TOPO TA cloning kit (Invitrogen) and sequencing reactions performed by GATC Biotech (Konstanz, Germany). Chromatograms were analysed with 4Peaks (http://www.mekentosj.com/4peaks). BLAST2 (http://www.ebi.ac.uk/blast2/nucleotide) was used for similarity searches whilst the program CLUSTALW (http://www.ebi.ac.uk/clustalw) was used for alignments. MEGA 3 (Kumar *et al*. 2004) was used for phylogenetic analysis.

Culturing assays

For culturing assays, adult individuals were prepared from field samples (leaf galled shoots of *Vitis vinifera* ssp.) and surface sterilized as described above. Each sample included 20-40 individuals. Samples were then transfered to fresh tubes containing 500 μl of phosphate buffer (20 mM KH_2PO_4, 30 mM Na_2HPO_4) and homogenized. The homogenates were streacked on sterile nutrient agar and on tryptic soy agar plates with five replicates each. Agar plates were sealed with parafilm and incubated for 24h at 25°C. Plates were evaluated the following day and single colonies were picked for further proliferation and examination. Eggs of grape phylloxera were also tested for the presence of bacteria.

Furthermore, tissue samples of leaf galls and ungalled leaves of *Vitis vinifera* ssp. were tested for the presence of bacteria, preparing small tissue pieces, washing them in 70% Ethanol and phosphate buffer and streaking homogenates on agar plates the same way it was done for insect tissues described above.

Standard descriptions and metabolic tests were applied according to „Bergey's manual of Determinative Bacteriology“ (Bergey *et al*. 1994) in order to describe the characteristics of the cultured bacteria. Broth medium cultures were started from single bacteria colonies for DNA extraction and further examination.

Results

Ribosomal DNA amplification and sequencing

Molecular methods were applied to search for the presence of microorganisms in grape phylloxera. Using specific PCR primers on total DNA extracted from adult phylloxera (see experimental procedures), fragments of bacterial origin were systematically amplified, cloned and sequenced. However, primers designed to specifically amplify fungi 18S rDNA sequences always failed to yield any amplified product.

16S rDNA fragments were amplified having the expected size of c.a. 900 base pairs for primers *16seqF1* and *16seqR1* (see experimental procedures), though unspecific PCR byproducts of smaller size were frequently co-amplified (Fig. 4). After analysis of the first sequences obtained, primers *pagF* and *pagR*, were specifically designed for *Pantoea agglomerans*. 16S rDNA amplification which systematically resulted in single highly spe-

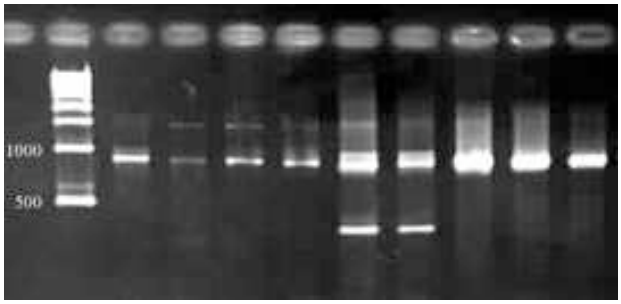


Figure 4: Amplification of 16S rDNA fragments with primers *16seqF1* and *16seqR1* in various populations of *D. vitifoliae* and bacterial isolates of *D. vitifoliae* (from left to right: GH, KI, JR, GW, BI, BI2, *in vitro* cultured bacteria BI, *in vitro* cultured bacteria BIE, control sample AF). Besides the bacterial 900 bp fragment, unspecific products of smaller size were partially amplified in some cases.

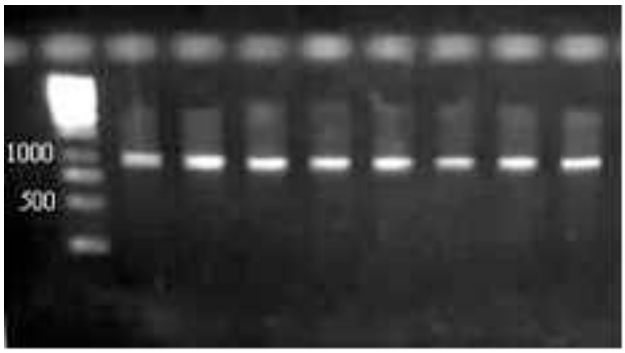


Figure 5: Amplification 16S rDNA fragments with primers *pagF* and *pagR* in various populations of *D. vitifoliae* (from left to right: BI, BI2, BIE, GW, GWE, FR, FR2, RAS). A single band of ca. 1000 bp was amplified.

cific fragments of about 1000 bp (Fig. 5). 35 to 40 cycles were usually needed to obtain clearly visible fragments, whereas fragments from the control aphid samples corresponding to *Buchnera aphidicola* 16S rDNA were usually detected after only 30 cycles.

Analysis of sequences obtained for the amplified 16S rDNA fragments revealed the presence of an Enterobacteriaceae in *D. vitifoliae*, closely related to *Pantoea agglomerans*. Sequences exhibited 99-100% similarity to GenBank sequences AF157694, AB004757 and AF130946. Occasionally, other bacterial species were identified but only once and only in single populations and were therefore considered transient or accidently uptaken by the insect and were not further analysed. *Pantoea agglomerans* 16S rDNA was identified in adult individuals, and also in samples of parthenogenetic eggs prepared from leaf galls. Sequences of *P. agglomerans* associated with *D. vitifoliae* were deposited in GeneBank (see table 14). No *Buchnera* DNA was detected in any phylloxera sample analysed. Sequencing of the corresponding fragments from control samples from *A. fabae* and *M. persicae* resulted in the detection of *Buchnera aphidicola* sequences and validated the methodological approach.

Table 14: *D. vitifoliae* populations tested for *P. agglomerans*, GeneBank accession numbers are given for published 16S rDNA sequences

Sample	Collection site	Sampling date	Host plant (variety)	GeneBank accession number
BI	Bingen, population 1 Germany, 8°00'E49°57'N	07/2001	Teleki 5C (*V. berlandieri* x *V. riparia*)	AM050138
BI2	Bingen, population 2, Germany, 8°00'E49°57'N	07/2003	Teleki 5C (*V. berlandieri* x *V. riparia*)	AM051062
BIE	Bingen, egg sample, population 2, Germany, 8°00'E49°57'N	07/2001	Teleki 5C (*V. berlandieri* x *V. riparia*)	AM050139
GW	Geilweilerhof, Germany, 8°03'E49°15'N	06/2003	Teleki 5C (*V. berlandieri* x *V. riparia*)	AM050140
GWE	Geilweilerhof, egg sample, Germany, 8°03'E49°15'N	06/2003	Teleki 5C (*V. berlandieri* x *V. riparia*)	AM050141
GH	Gundelsheim, Germany, 9°10'E49°17'N	07/2001	Teleki 5C (*V. berlandieri* x *V. riparia*)	Sequence unpublished
KI	Kientzheim, France 7°20'E48°10'N	07/2000	Couderc 3309 (*V. riparia* x *V. rupestris*)	Sequence unpublished
RAS	Rouffach, France, 7°17'E47°57'N	07/2003	Teleki 5C (*V. berlandieri* x *V. riparia*)	AM051064
FR	Fanjeaux, France, 02°01'N43°11'E	07/2004	110R (*V. riparia* x *V. rupestris*)	AM050142
FR2	Fanjeaux, 2nd sampling site, France, 02°01'N43°11'E	07/2004	110R (*V. riparia* x *V. rupestris*)	AM051063
JR	James River, USA, 78°29'W37°46'N	06/2000	*Vitis arizonica*	AJ746342
AF	Aphis fabae, control sample, greenhouse	08/2001	*Pisum sativum*	Sequence unpublished
MP	Myzus persicae, control sample, greenhouse	08/2001	*Vitis vinifera* cv. Bacchus	Sequence unpublished

Genetic variation in 16S rDNA sequences of associated bacteria

16S rDNA sequences were obtained from 9 different phylloxera samples and deposited to GenBank (see table 14). A total of 506 nucleotides were unambiguously read for these 9 samples and were aligned using ClustalX (Thompson *et al.* 1997). All sequences were identical for the aligned portion except BI2 and RAS (see table 14), which differed from the other sequences by 2 and 1 nucleotide positions respectively (Fig. 6) . Minor genetic variation is thus present in populations of associated bacteria of grape phylloxera. The phylloxera originated sequences were also aligned with corresponding sequences from other related enterobacteriacea and a phylogenetic tree was constructed using the Neighbor Joining algorithm (Fig. 7) . The highly supported monophyly of a cluster containing our phylloxera derived sequences and *Pantoea agglomerans* sequences available in GenBank confirm that all sequences derived from different phylloxera samples do correspond to *Pantoea agglomerans* bacteria associated with both phylloxera adults and eggs.

Culturing assays

The microorganisms harboured by *D. vitifoliae* revealed to be culturable on simple bacterial media like nutrient agar or tryptic soy agar. Only one single bacterial species grew on the plates, when insects were surface sterilized as described in Experimental procedures. This confirms that washing steps prior to homogenization were successful in surface sterilisation of the insect material. Bacteria grown in this assay revealed to be fully viable and not obligate biotroph, which is in contrast to other endosymbiotic bacteria.

Moreover, bacteria cultures were successfully established from samples of adult phylloxera of different populations, egg samples and also from tissues of grape phylloxera leaf galls. No bacteria could be cultured from non-infected (not galled) *Vitis* leaf tissue samples.

Bacteria exhibited morphological and biochemical characteristics of the family Enterobacteriaceae. Colonies were entire and yellow pigmented, consisting of gram-negative, short bacilli, which were oxidase negative, catalase positive, citrate positive, glucose and other carbohydrates were catabolized with the production of acid but no gas production (Kortekamp 2004). In dual cultures of *P. agglomerans* isolated from grape phylloxera revealed antagonistic effects against 13 fungi species and seven bacteria species (Lee 2005).

Discussion

As shown in this study, molecular methods have been successfully applied in order to identify associated bacteria in *D. vitifoliae*. A single bacterial species of the genus *Pantoea* was found in all phylloxera populations tested from different geographic locations.

We confirmed the absence of mycetomes and endosymbiotic *Buchnera* species in *D. vitifoliae* with histological and molecular genetic methods (Vorwerk *et al.* 2005). Molecular

```
consensus       ATTCCGTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGC 60
RAS             ATTCCGTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGC 60
BI2             ATTCCGTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTCGCATCGAATTAAACCACATGC 60
                ************************************ ********************

consensus       TCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCC 120
RAS             TCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCC 120
BI2             TCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCC 120
                ************************************************************

consensus       CCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACTCCTCAAGGGAACAACCTCCAA 180
RAS             CCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACTCCTCAAGGGAACAACCTCCAA 180
BI2             CCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACTCCTCAAGGGAACAACCTCCAA 180
                ************************************************************

consensus       GTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT 240
RAS             GTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT 240
BI2             GTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT 240
                ************************************************************

consensus       TCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGA 300
RAS             TCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGA 300
BI2             TCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGA 300
                ************************************************************

consensus       TCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCAAGCCTG 360
RAS             TCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCAAGCCTG 360
BI2             TCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCAGGCCTG 360
                ************************************************** *****

consensus       CCAGTTTCAAATGCAGTTCCCAGGTTAAGCCCGGGGATTTCACATCTGACTTAACAGACC 420
RAS             CCAGTTTCAAATGCAGTCCCCAGGTTAAGCCCGGGGATTTCACATCTGACTTAACAGACC 420
BI2             CCAGTTTCAAATGCAGTTCCCAGGTTAAGCCCGGGGATTTCACATCTGACTTAACAGACC 420
                ********** ***************************************

consensus       GCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGC 480
RAS             GCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGC 480
BI2             GCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGC 480
                ************************************************************

consensus       GGCTGCTGGCACGGAGTTAGCCGGTG 506
RAS             GGCTGCTGGCACGGAGTTAGCCGGTG 506
BI2             GGCTGCTGGCACGGAGTTAGCCGGTG 506
                *************************
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Figure 6: Single nucleotide polymorphisms were identified in two samples of associated bacteria from, RAS and BI2, when comparing them to all other *P. agglomerans* 16S rDNA samples sequenced (indicated in the figure as "consensus"). The polymorphisms indicate genetic variation of associated bacteria among different populations of *D. vitifoliae*.

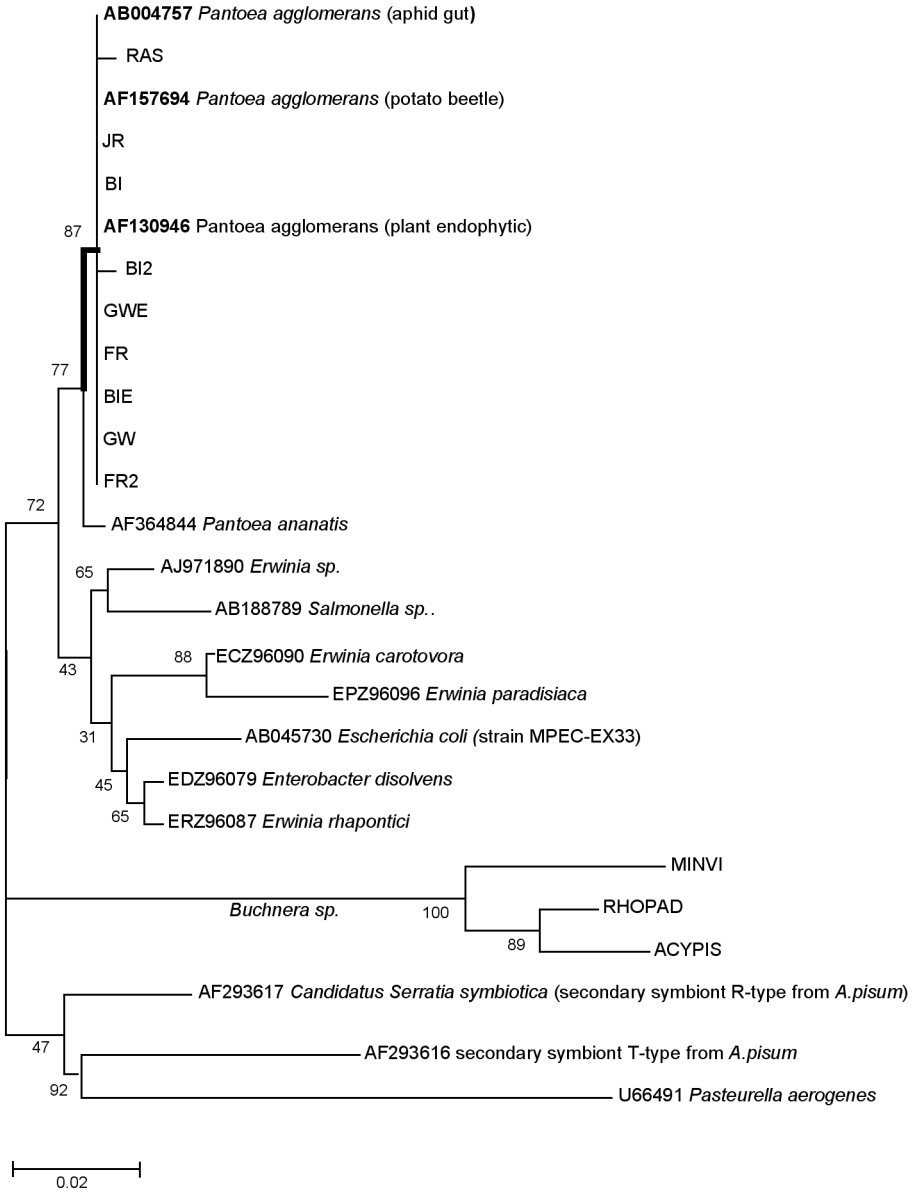

Figure 7: Neighbor Joining tree using Kimura two parameters distance showing the phylogenetic relationships for the 16S rDNA sequences obtained from the different *D. vitifoliae* samples along with some other Enterobacteriaceae sequences available in GenBank. Phylloxera derived sequences are indicated as in Table 13. Branch leading to *Pantoea agglomerans* related sequences has been thickened. Sequences from other Enterobacteriaceae are indicated by their accession number along with the species name. Sequences from primary (*Buchnera* sp. branch) and secondary aphid symbionts have been included. MINVI, *Mindarus viminalis*; RHOPAD, *Rhopalosipum padi*; ACYPIS, *Acyrthosiphon pisum*). The bar scale indicates number of nucleotide differences per site. Numbers close to tree nodes indicate percentage bootstrap support for that node after 500 pseudoreplicates.

methods as here employed were based on common phylogenetic studies of *Buchnera* species in Aphididae with modifications of PCR conditions to identify low copy numbers of bacterial sequences present in *D. vitifoliae*. The design of more specific primers for *Pantoea* species in this study makes it possible to test more precisely for the presence of *Pantoea* species in further populations of *D. vitifoliae* and other related phylloxerids.

The type of association between *Pantoea* ssp. and grape phylloxera has without doubt to be regarded as being radically different from the association of *Buchnera* and aphids of the family Aphididae, which ingest phloem sap, have mycetocytes and require essential amino acids (Dixon 1998). *D. vitifoliae* is monophagous, feeding only on *Vitis* species in closely defined habitats. The food uptake of *D. vitifoliae* clearly differs from the food uptake of members of the Aphididae. Grape phylloxera penetrate through several cell layers to suck the cell content of parenchymatic tissues. They ingest a protein rich diet and do not need bacterial support for synthesis of essential amino acids or proteins as do Aphididae which feed on phloem sap. A symbiotic association with *Buchnera* is therefore not essential, which was already stated by Buchner (1965) and Baumann *et al.* (1995).

Grape phylloxera have evolved strong specialization by focusing on one host plant species. Complex interactions between host plant, insect and infecting bacteria may therefore have been established over a long time period. The presence of a single bacterial species, closely related to *Pantoea agglomerans*, in adult parthenogenetic individuals of *D. vitifoliae*, their eggs and in tissue samples of leaf galls suggests that the association between the bacteria and the insect might more stable than a mere transient infection. However, at this point of our research the impact of the interaction between *P. agglomerans* and *D. vitifoliae* remains unclear and two possible postulations can be stated. (1) The bacteria may profit through improved living conditions within a phylloxera induced leaf galls and infection of the aphid occurs by accident with no benefit for the insect (transient microorganisms). (2) A stable association could have developed between bacteria and grape phylloxera over time, resulting in mutualistic benefits (symbiosis). *P. agglomerans* may profit from improved living conditions provided by the leaf gall and *D. vitifoliae* may benefit from the antagonistic potential of the bacteria (Dillon & Charnley 1995, Ishimaru *et al.* 1988, Wright *et al.* 2001). *In vitro* assays with *P. agglomerans* strains isolated from *D. vitifoliae* showed inhibition of microbial growth and activity (Lee 2005). The bacteria may be a part of the protection system the insect benefits from in its feeding habitat, complementing the physiological properties of the leaf gall. However, *in vitro* culturing assays showed that the bacteria are fully viable, which suggests that the association between *D. vitifoliae* and *P. agglomerans* is not as firm as associations between aphids and *Buchnera* endosymbionts.

Pantoea agglomerans belongs to the family of Enterobacteriaceae in the gamma subdivision of proteobacteria. It is therefore closely related to *Escherichia coli* species and *Buchnera aphidicola,* which belong to the same group. The names *Enterobacter agglomerans* and *Erwinia herbicola* are both used for *Pantoea agglomerans*. Recent re-evaluations in

systematics reveal new habitats and relationships between plant pathogenic *Erwinia* species and insects, or even mammal pathogen species. *Pantoea agglomerans* is a gram-negative rod shaped bacteria with high genetic plasticity. The bacterial species are commonly present in a wide range of habitats, such as plant surfaces, seeds, water, in insects and in mammals (Gavini *et al*. 1989). *P. agglomerans* has been found to induce galls by producing elevated levels of IAA on various plant species (Best *et al*. 2004). It has further been shown to produce antibiotics (Wright *et al*. 2001) and antifungal phenolics (Dillon & Charnley 1995).

Pantoea species are found in other phytophagous insects. The thrips, *Frankliniella occidentalis* (Pergande), was examined by De Vries *et al*. (1995, 2001) and *Pantoea* was identified as the main species in the hindgut. The microbial flora is thought to support the microbial defense against pathogens by producing antimicrobial compounds. Another form of associated *Pantoea* bacteria was found in *Schistocerca gregaria* (Dillon & Charnley 1995, Dillon *et al*. 2000). These locusts need to ingest the bacteria from food plants in every generation. However the association is stable and the bacteria produce antifungal phenolic substances that protect from gut infections. *P. agglomerans* was also identified in the aphid species *Acyrthosiphum pisum* by Harada *et al*. (1996, 1997), who suggested a new species name: *Erwinia aphidicola*. The sequences identified in this study show high similarity to the sequences identified for *P. agglomerans* in *D. vitifoliae* (Fig. 7).

Although there have been some hints in this experiment, maternal transmission cannot be confirmed yet. 16SrDNA sequences of *P. agglomerans* are found in parthenogenetic adults and eggs of the same population, suggesting that some trans-ovaric infection must occur. Up to now, it is still open, whether we have to deal with "location"-specific or phylloxera-specific strains of *P. agglomerans*. Studies are underway to elucidate the mode of association of the bacteria in the life cycle of *D. vitifoliae* using *in vitro* dual culturing assays and individuals of all life stages of grape phylloxera need to be tested for the presence of microorganisms.

The role of *P. agglomerans* in grape phylloxera is still to be defined. Symbiotic and associative bacteria may play a role in insect fitness and adaptation to different host plants and lead to the formation of new biotypes. In this context it is relevant to mention the emergence of new and aggressive strains of grape phylloxera reported in the last ten years in Europe and elsewhere (Boubals 1994, Forneck *et al*. 2001, Kocsis *et al*. 1999). Whether *Pantoea agglomerans* plays a role in the evolution of phylloxera traits involved in the insect-plant interactions has yet to be investigated. The elucidation of further details concerning grapevine-insect-microbe interactions may well be a step towards development of a new ecological plant protection approaches against grape phylloxera.

7 Application of current *in situ* hybridization techniques for grape phylloxera (*Daktulosphaira vitifoliae* Fitch) and grapevine (*Vitis* spp.)[6]

Abstract

In situ hybridization and *in situ* PCR directly localize specific DNA and RNA sequences in tissues. To exactly focus on the processes occurring on cell- or tissue level, *in situ* techniques can be efficiently employed. Recent advances in viticultural research pertaining the fields of genomics and proteomics are likely to employ these techniques to link DNA- or mRNA sequence information to physiological traits and processes occurring in the grapevine. In this paper, we would like to present a range of possibilities for *in situ* techniques that can be applied in grapevine research. Two examples will be given for illustration. Moreover, key steps of the techniques are discussed, which may be helpful to researchers aiming to employ *in situ* hybridization or *in situ* PCR.

Introduction

With the help of *in situ* hybridization techniques and nucleic acid probes target DNA sequences can be localized in their native tissue or cell environment. The combination of histological methods with *in situ* PCR techniques allows for detecting and quantification of specific DNA or RNA sequences in a sample. The recent development of non-radioactive methods for nucleic acid labelling simplifies the application of this technique which is employed for a range of biological and ecological scientific questions: e.g. description of bacterial species in biofilms (Okabe *et al.* 1999), the composition of ecosystems (Zheng *et al.* 1996), the localization of viral material in plant tissues (Singh & Nie 2002) or the localization of secondary symbionts in insects (Harada *et al.* 1996). In viticultural sciences, first approaches were made by Haas *et al.* (1994) and Haas & Alleweldt (2000) employing *in situ* hybridization techniques for karyotype studies of *Vitis vinifera* (L.) and by Sohier *et al.* (1998) detecting bacteria in fermentation processes of wine.

This article presents applications of *in situ* techniques for current grapevine research. *In situ* hybridization was successfully applied to localize associated bacteria within tissue sections of grape phylloxera (Vorwerk *et al.* 2007a). Furthermore, activity tests of specific genes involved in host-parasite interactions in root tips of grapevine were modelled using new *in situ* RT-PCR techniques. These two examples together with a critical discussion of key steps of the techniques may be helpful to researchers aiming to employ these techniques.

[6]Vorwerk, S., Sonntag, K., Ottaviano, F., Blaich, R. & Forneck, A. (2007c) Application of current *in situ* hybridization techniques for grapevine (*Vitis* spp.). *Vitis*, submitted.

Materials and Methods

7.1 Example 1: Localization of starch synthesis involved genes in phylloxera infected roots

Introduction

Grape phylloxera induce complex interactions with their host, establishing galls on leaves and roots as permanent feeding sites. Due to permanent stimulation, the feeding sites accumulate starch globules (Forneck *et al.* 2002). In this experiment, the activity of genes involved into starch synthesis in phylloxera-infected roots was examined using RT *in situ* PCR with three specific primer pairs amplifying 350-400 bp of the invertase and ADP-glycose-pyrophosphorylase gene (table 15). The use of cDNA in this case as template for hybridization is advantageous since the activity of a specific gene can be monitored by evaluating the strength of the hybridization signal. Results of the first strand synthesis revealed high cell activity around the penetration site. Second strand synthesis, employing two specific primer pairs in RT *in situ* PCR on the samples, revealed clear and strong signals of gene activity radially spread around the feeding site, but not at the opposite side of the pericycle. Both primer sets revealed stronger hybridization signals in root tips of early infection stages than in the older ones. This may be due to the fact, that older infected roots tips might already have declined cell activity and that the PCR reaction might have been more difficult to perform in partly lignified cells. Non-infected root tips, employed as a control reaction did not show any hybridization signal with none of the primer sets, but autofluorescence was clearly visible on the exodermis.

Methodology

Teleki 5C (*V. berlandieri* x *V. riparia*) rootstocks were propagated from two-node dormant cuttings and infected with eggs of a parthenogenetic grape phylloxera population collected at Bingen, Germany (Forneck *et al.* 2000). Nodosities of three different stages were collected (Fig. 8) and stored at -20 ˚C. Root-tips of non-infected rootstocks T5C were employed for control reactions. Fixation, embedding and sectioning was performed according to the descriptions of Example 1 applying the following modifications: Serial sections were adjusted to 8 μm. Pepsin (2 mg/ml in 0,01 M HCl for 60 minutes at 37 ˚C) was employed instead of proteinase K in order to make cell walls permeable for the penetration of labelled probes. Since this experiment was based on RNA, no RNAse was employed for digestion, but DNA was digested using 20 U DNAse in 40μl of PCR buffer per sample. Special care was taken to work under RNAse free conditions, reagents were prepared using DEPC-treated water (Applichem, Heidelberg), slides and experimental material were autoclaved and benches were treated with RNAse-OFF (Applichem). cDNA was synthesized using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (MBI Fermentas) with an oligo dT_{13} primer (0,5

μg/μl). The RT *in situ* PCR was conducted using 25 mM $MgCl_2$, 2 mM dNTPs, 20 pM/μl of each primer, 10x PCR buffer, 5U/μl Taq-Polymerase (Invitrogen) and 20% Roti-stab (Roth, Karlsruhe) in a total volume of 50 μl. Special frames for *in situ* PCR (Eppendorf) were used to keep the PCR mix sealed on the sample during the PCR reaction. Ten cycles of 'touch-down PCR' (annealing temperature 50-45 ˚C) were applied, followed by 20 cycles of standard PCR (annealing temperature 45 ˚C) (see Sonntag 2005 for further details). Cycles for *in situ* PCR were extended to 60 seconds each. Samples were very gently dipped into 2x SSC buffer (in petri dishes) in order to eliminate unbound primers prior to microscopic examination.

7.2 Example 2: Localizing insect-associated bacteria inside grape phylloxera

Introduction

Grape phylloxera is one of the worldwide most important pests in viticulture. Newly upcoming aggressive grape phylloxera biotypes, have renewed interest in this pest. In connection with this news, phylloxera-associated bacteria of the genus *Pantoea* were identified to be involved in the parasite-host interaction (Vorwerk *et al*. 2007a). In order to elucidate their function and transmission specific 16S rDNA probes were hybridized on ultra-thin sections of adult leaf gall grape phylloxera. Signals were detected inside the salivary pump of the insect and also within eggs inside the insect body (Fig. 8). Positive control samples revealed signals within the gut of *A. fabae* and *M. persicae*, pointing to the presence of *Buchnera* bacteria. Using the same probe concentration, control samples presented notably stronger signals than grape phylloxera samples. Negative controls, employing a hybridization mix without 16S rDNA probes did not reveal any signal.

Methodology

Four leaf galling grape phylloxera populations were collected from different locations in Europe (Bingen, Gundelsheim (GER), Rouffach (F), Turin (I), including samples of *Aphis fabae* (Scopoli) and *Myzus persicae* (Sulzer) bearing endosymbiotic *Buchnera* as positive controls. For each sample, 50 adult aphids were prepared from leaf galls and washed shortly in 70% ethanol. Insect samples were fixed in Johansen solution and formalin-propionic acetate and further cleared through an ethanol-xylene series before being embedded in paraffin at 65˚C. Serial sections were adjusted to 6-7 μm for pre-cooled insect tissue samples. Sections were deparaffinized through a xylene-ethanol series (Lillie *et al*. 1965) prior to each hybridization.

For bacteria a specific 54 bp oligonucleotide probe, developed from *P. agglomerans* sequenced fragments (Vorwerk *et al*. 2007a), 5'end labelled with digoxygenin, was applied.

Table 15: Probes and sequences employed for *in situ* techniques

Study	Sequence name	Sequence 5'-> 3'
Endosymbiotic bacteria of grape phylloxera	54 bp oligonucleotide probe for Pantoea agglomerans (PAG)	CGC ATA CAA AGA GAA GCG ACC TCG CGA GAG CAA GCG GAC CTC ACA AAG TGC GTC
	54 bp oligonucleotide probe for Buchnera aphidicola (BAP)	TTT ATA CAA AGA GAA GCA AAT CTG CAA AGA CAA GCA AAC CTC ATA AAG TAA ATC
Starch synthesis in phylloxera infected roots	Invertase 1, left*	GCC CAG TGT ATC ACA AGA TT
	Invertase 1, right*	GGA GAT GAA GCC ACT CTA TG
	Phyrophosphorylase 3, left*	TGA AAG CTA TGA AGG TCG AT
	Phyrophosphorylase 3, right*	CGG TCA TAG AAG CTG AAA TC

* these primers were also tested on chimeric leaves of Pinot meunier (see Example 3)

Another 54 bp 16S rDNA fragment hybridizing to *Buchnera* but not to *Pantoea* was chosen for application to the control samples (table 15). After deparaffinization, samples were treated with RNAse A (100 μg/ml in 2x SSC (saline-sodium citrate buffer)) for 30 minutes at 37 ˚C and then gently washed in 2x SSC to eliminate RNA templates and prevent non-specific bindings. Samples were further treated with Proteinase K (2,5 μg/ml in 2x SSC) for ten minutes in order to make cell walls permeable for the labelled probe and then fixed in proteinase K stop buffer and 4% formaldehyde. The hybridization mix consisted of 50% formamide, 10% dextransulphate, 1ng/ml labelled DNA probe, 250 ng/ml herring sperm DNA, 1,25% SDS (sodium dodecyl sulphate buffer) in 2x SSC. 40 μl were applied and samples were covered with coverslips and placed in humid chambers. The hybridization reaction comprised a denaturation step of ten minutes at 95 ˚C and the hybridization step itself overnight (ten to twelve hours) at 37 ˚C. After hybridization, tissue sections were washed twice in washing buffer (50% formamide in 2x SSC) at 42 ˚C for three minutes before being incubated in detection buffer containing 2 μg/ml anti-digoxygenin-antibodies conjugated to either fluorescein or rhodamin (both dyes were tested). Finally, slides were treated with 40 μl DABCO antifading solution (Sigma) and covered with new coverslips for microscopic examination. Samples were examined directly after hybridization using epifluorescence microscopy with an Axioplan microscope equipped with an UV-lightsource and UV-filters (exitation 450-490 nm, FT 510, LP 520). Results were documented using digital image processing (Axiocam, Carl Zeiss) and AxioVision 3.1.

Results and Discussion

In situ hybridization techniques were successfully applied in two different fields of grapevine research. The connection of molecular techniques with direct localization and visualization in the tissue sample make *in situ* techniques advantageous compared to other methods of investigation.

Which type of *in situ* technique is chosen, depends on the particular question of interest. Direct *in situ* hybridization is the easiest and safest technique, but requires a sufficient copy number of target sequences. The combination with PCR techniques allows good detection results even when only low copy numbers of the target sequence are present. An additional possibility of quantification is given by RT PCR techniques and the use of cDNA.

Every *in situ* experiment consists of three important aspects: the histological preparation of samples, the choice of adequate probes and the hybridization technique itself.

Careful histological preparation of samples, accurate embedding and high-quality sectioning significantly influence the results of hybridization experiments. For sample fixation, FAA (formaline and acetic acid), FPA (formalin-propionic acetate) or Karnovski solution (see Lillie *et al*. 1965) are commonly used. In the here presented experiments, FAA and FPA fixation worked equally, only Karnovski solution was found to show insufficient results, possibly due to the very strong binding structures produced. When working with lignified plant tissues (Lillie *et al*. 1965) or also insect tissues (containing trachea and chitinized structures) (Fukatsu *et al*. 1998), careful elimination of air vesicles in of the samples is very important to obtain intact sample structures for exact signal interpretation and good microscopical documentation. Paraffin embedded samples provide the best basics for *in situ* hybridization. Embedding procedures are simple, fast and non-toxic. Relatively thin sections can be produced which stick well to silane-coated slides (2% aminopropylethoxysilane in acetone), so that they can be treated through a number of buffers during *in situ* hybridization. As noted in all three experiments, it is helpful to pre-cool paraffin blocks before sectioning. Plastic embedding produces thinner slides, however tissue structures can be affected (Osamura *et al*. 2000) and become useless for nucleic acid *in situ* experiments.

The length of a probe for the detection of specific sequences inside a tissue may range from 20 to 1000 base pairs. The longer, the more specific, but very long sequences are difficult to penetrate through cell walls (Nuovo 1996) Therefore, probes should be kept as short as possible without loosing specifity. Specifity of probes can be checked by using online alignment services in common nucleotide databases. Ribosomal DNA or RNA specificity can be checked by using the CHECK_PROBE program of the Ribosomal Database Project RDP (rdp.cme.msu.edu/html). 5‘ labelling is recommended since it rarely interferes with the binding of probe and target sequence. Probes may be labeled with biotin or digoxygenin, which serve as reporter molecules and can be detected via antibodies. The antibodies then are conjugated to fluorescent molecules like fluorescein or rhodamin. Antibodies can also be

Table 16: Choice of control experiments for *in situ* hybridization and *in situ* PCR

Control experiment	Result
No-probe control	Use of dd H_2O instead of oligonucleotide probe, no signal should be detected
RNAse/DNAse digestion	After digestion of the target sequences (RNA/DNA) no hybridization should be possible
Housekeeping control probe	Using a universally active gene sequence as a target
Housekeeping control "aphids and endosymbionts"	Using aphid tissue that is proved to contain endosymbiotic DNA or alternatively using a universal bacterial probe which hybridises with most bacterial 16S ribosomal sequences (EUB 338, Amann *et al*. 1990)

conjugated to alkaline phosphatase that catalyses a non-fluorescent reaction (NBT-BCIP). In previous experiments, no differences were found between directly labeled and digoxygenin-labeled probes (data not shown). High levels of autofluorescence may be present in some tissue samples, especially in tissues of lignified root tips, due to the presence of ligning and phenolics-rich cell walls, but also in grape phylloxera adult bodys, which contain chitin and high amounts of fat. This does not need to be disadvantageous. The tissue structure will be easily seen in the background and detectable molecules will be easily localized. The labelling or detection mode should therefore be chosen to contrast well with the background of the sample. Rhodamin was observed to contrast well in both root and grape phylloxera tissue samples.

For clear and specific detection of hybridization signals, the right pre-treatments of the samples are required. A nuclease treatment to eliminate either RNA or DNA is useful to reduce background signals resulting from unspecific bindings. To facilitate permeabilization of the tissue, proteases like pepsin or proteinase K may be employed. Here, concentrations and application times need to be tested before starting the experiment. Furthermore, stringent conditions are necessary to prevent false hybridization signals. Stringency factors include hybridization temperature, addition of formamide, salt concentrations and time factors (Baldino *et al*. 1989). Additionally, Denhard's solution and sonicated herring sperm DNA were employed for the detection of 16S rDNA (see Example 1) as competitive ingredients and probes were let to hybridize for at least 12 hours over night. Finally, appropriate control experiments are essential. Table 16 shows different possibilities for control experiments.

Working with nucleic acids in woody perennials as grapevine often causes difficulties due to high amounts of interfering substances within tissues. The extraction of DNA from green parts of grapevine may be limited by the presence of polyphenols, whereas roots may contain high amounts of starch and sugars (Iandolino *et al*. 2004). This is especially crucial for the isolation of RNA, in order to produce cDNA for example, if the quantity of isolated material is not high enough. In this case *in situ* techniques may be the methods of choice, since cDNA could be produced and then further processed without loss on the sample tissue.

Furthermore, *in situ* techniques should be applied, if direct localization of specific sequences is essential. As already applied for the detection of lactic acid and other bacterial species in vine (Sohier *et al.* 1998, Stender *et al.* 2001), the use of differently labelled probes would make it possible to identify different DNA products simultaneously in a tissue and even monitor their development over time (Amann *et al.* 1995, Amann *et al.* 1996). This could be a useful application when thinking of monitoring growth stages or also different stages of infection in pest and disease diagnostics. Also for karyotyping, *in situ* techniques represent a solid basis, especially for grapevine, possessing very small chromosomes, as shown by Haas and Alleweldt (2000). Moreover, first *in situ* techniques have recently been established for the detection of chimeric tissues and represent a further interesting field of application for *in situ* techniques in grapevine.

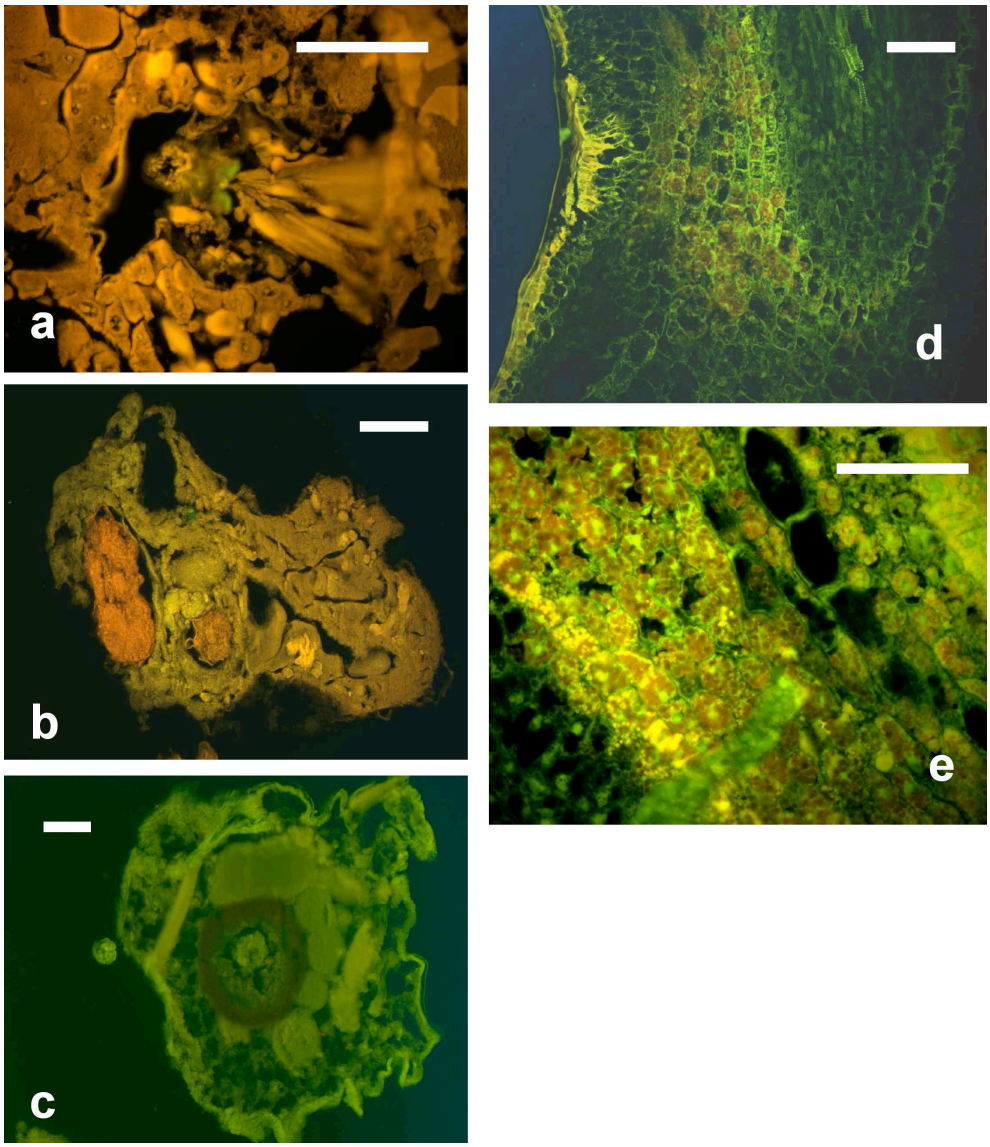

Figure 8: a-e: 16S rDNA of grape phylloxera associated bacteria detected with a fluorescein-labelled oligonucleotide probe in the salivary pump of an adult parthenogenetic grape phylloxera (transversal cross-section) (a). 16S rDNA of grape phylloxera associated bacteria detected with a rhodamin-labelled oligonucleotide probe in the eggs inside an adult parthenogenetic grape phylloxera (longitudinal section) (b). 16S rDNA of *Buchnera aphidicola* detected with a rhodamin-labelled oligonucleotide probe in specific cells near the gut lumen (mycetocytes) of *Aphids fabae* (cross-section) (c). *In situ* RT-PCR in nodosities of phylloxera infected root material of Teleki 5C, employing primers for invertase and pyrophosphorylase. Gene activity was monitored around the feeding site (d, e). Bars represent 100 μm.

8 Conclusion

This thesis aimed to elucidate the genetic and biological characteristics of asexual grape phylloxera populations. A combined approach, employing molecular methods, phenotypic evaluation and histochemical techniques elucidated various aspects of the subject. Analysis of field-sampled populations ascertained that grape phylloxera in Europe reproduce asexually, although a high quantity of genetic variation was detected within the populations (chapter 3). Intraclonal variation was described in detail using a multilocus marker system on parthenogenetic single founder lineages of this insect (chapter 4). The high level of genetic variation detected clearly derived from genetic variation within the grape phylloxera genome and not from contaminating foreign DNA, although bacteria closely related to *Pantoea agglomerans* were identified to be associated with the insect (chapter 6, 7). Intraclonal variation leads to a high potential of adaptation to new hosts or environmental conditions, as demonstrated with performance data on single founder lineages of grape phylloxera (chapter 5).

Since its first introduction a hundred and fifty years ago, grape phylloxera populations have spread across all European wine growing regions. The introduction of resistant rootstocks efficiently contained the damage of this aggressive insect pest and today the presence of grape phylloxera populations is mainly limited to habitats as abandoned vineyards, rootstock nurseries and few still own-rooted vineyards. Codominant microsatellite data suggest that asexual reproduction is predominant in these populations, revealing similar population genetic parameters as propounded in studies of other asexual aphid species (Delmotte *et al.* 2002, Sunnucks *et al.* 1997, Vorburger *et al.* 2003). As other asexual aphids, parthenogenetic grape phylloxera may have changed their mode of reproduction to asexuality due to milder climates, which make it possible for parthenogenetically produced eggs or first instars to overwinter (Phillips *et al.* 2000, Shufran & Wilde 1994) or other still unknown factors.

The genetic structure of these populations reflects the specific characteristics of their habitats. No overlapping of genotypes was found among collection sites, due to the absence of host-alternation and physically limited dispersal ability. Nontheless, genetic diversity is present within habitats and multiple samplings within one season revealed that mutations, eventual rare sexual reconbination and selection events lead to rapid clonal turnover. This was confirmed by a further experiment, analysing in detail the intraclonal genetic variation of parthenogenetic single founder lineages with a multilocus marker system. Interestingly, not only a high level of genetic variation was determined within the lineages, but it was also found that mutations were generated constantly in every generation. No accumulation of specific mutations was detected throughout the generations as described in the models of mutational meltdown by Lynch *et al.* (1993). Only a few single mutations were traced throughout the generations analysed. Whether these are related in some manner to the adaptation of these clones needs to be further inverstigated. So far, only few approaches have been

made to identify adaptation-related markers (e.g. Corrie *et al.* 2003). First attempts were made in this thesis to monitor the transfer of parthenogenetic single founder lineages to new host plants by means of simple measurable performance parameters in combination with the multilocus marker system previously used. Two markers were identified and clearly assigned to the adapted lineages. Certainly, the use of AFLP markers implies that these two markers were most probably located in non-coding regions. It is therefore necessary to further elucidate the correlation of these markers with concrete adaptational traits. The markers need to be sequenced and tested for linkage on the host and then converted into SCAR markers for the screening of further populations. Defining biotypes and their potential for aggressivity by evaluating both phenotypic performance data on single founder lineages on whole host plants in combination with genetic markers is useful and closer to „realistic“ conditions than the commonly applied „exised root assays“ (see Granett *et al.* 1987, Kocsis *et al.* 2002). If in future more adaptation-related markers are developed, rapid genetic tests will readily complement bioassays.

Marker systems need to be carefully chosen, in order to obtain the maximum of information to answer the question of interest. In this thesis, all molecular methods were therefore thoroughly adapted to the organism analysed, the specific question and the laboratory conditions. This involved the development of new codominant primer combinations (chapter 3, 6), a modified AFLP marker system employing alternative restriction enzymes and primers (chapter 4), but also the adaptation of *in situ* techniques, which had not yet been established for grapevine and grape phylloxera (chapter 7). Furthermore, the use of commercial kits revealed to be advantageous for some techniques, as for example DNA extraction, the cloning of fragments, but also the use of new high resolution gels, allowing to separate AFLP fragments and directly sequence them from the gel (chapter 4). Although techniques and compositions of commercial kits need to be critically asessed, the above-mentioned techniques revealed to be simple, time-saving and guarantied uniformity of resulting products.

Results of a marker analysis, however, need to be checked attentively, since the resulting data only represent a more ore less smaller fraction of the genome behind it. For example, employing a reduced set of microsatellite loci to grape phylloxera populations (chapter 3) leads to a less detailed distribution of genotypic classes in the dataset, compared to employing all six loci. Especially for the genotyping of field populations, it may be necessary to develop more microsatellite loci (Lin *et al.* 2006) or even to combine several marker techniques in order to receive the most confident information possible. The combination of microsatellite data with mitochondrial haplotype data (Corrie *et al.* 2002, Downie & Granett 1998) could be an interesting complementation. Moreover, AFLP fragments indicating same molecular weights were found to be not necessarily homologous in sequence (chapter 4). This fortunately does not have implications on the detection of genetic variation during direct comparison of single individuals, but may be relevant when developing specific single locus markers from AFLP datasets. This the reason is why correlation of these markers to a

very specific phenotype would be very important.

The presented approaches have renewed the information on the reproductive mode of grape phylloxera still dating from the second last century. Meanwhile, several variations of the grape phylloxera life cycle have been investigated and the occurence of asexually reproducing populations is widespread (Corrie *et al.* 2002). The genetic or environmental factors inducing these life cycle alterations have not been exactly defined yet. It would be interesting to monitor leaf- and rootgalling populations over a time period of several years to investigate how mainly clonally reproducing populations develop, how leaf- and rootgalling populations differ in genotypic composition and to what extent clonal turnover and cold winter periods might influence such a population.

The monophageous relationship between grape phylloxera and its host seems to be very intense, but also complex. The identification of specific genotypes for each habitat suggests that not only the host plant but the habitat on the whole seems to have great influence on the development of the insect. Presumably, most of the factors affecting this strong host-parasite relationship have not yet been identified. The detection of closely associated bacteria in this thesis may be one of these factors, its role however is still undefined. *In vitro* tests showed that some bacteria exhibit an antifungal acitivity by producing toxic substances. Whether this is of active use for the insect or the leaf gall remains to be understood. Although, the positive function of the toxin was though confirmed for other insects, for example the locust *Schistocerca gregaria* (Dillon & Charnley 1995, Dillon *et al.* 2000). Meanwhile, the transmission of these bacteria within parthenogenetic grape phylloxera has been confirmed (data not shown) and corroborates the localization of bacteria in eggs within adult grape phylloxera obtained by *in situ* hybridization assays. Moreover, specifically designed primers also revealed the presence of these bacteria in rootgalling grape phylloxera (data not shown), suggesting that these associated bacteria are closely involved in the life and performance of phylloxera on grapevine.

For asexually reproducing plants and other organisms, there exist models, tempting to describe characteristics and behaviour of asexual populations - the best-known are the „general purpose genotype“ model by Baker (1965) and the „frozen niche variation“ model by Vrijenhoek (1984). In studies on asexual aphid lineages, the „general purpose genotype“ was renamed „superclone“ (Vorburger *et al.* 2003). These superclones, for example an asexual genotype of *Myzus persicae* in Australia, revealed to occur in a broad environmental and geographic range. For European grape phylloxera, however, this phenomenon does not apply as shown in a study of codominant markers in this thesis, although Australian researchers affirm to have identified two grape phylloxera superclones (Corrie *et al.* 2002, Corrie & Hoffmann 2004). This may be due to very specific allocation structures of grape phylloxera in Australia. Do grape phylloxera thus rather belong to the highly specialized type of asexual organisms? They seem to be perfectly adapted to their specific habitat and the genotypes found in one habitat will not be found elsewhere. The potential for genetic changes and

adaptation always remains, at least for single clones within a population. The potential to develop aggressive biotypes which may migrate to cultivated vineyards nearby, is present in each population of this insect pest. This has already been reported for vineyards in Europe and elsewhere (Boubals 1994, Granett *et al.* 1985, Kocsis *et al.* 2002, Presser 1993), but was also observed by the author in Southwestern France, where grape phylloxera infestations had spread from wild growing rootstocks to the closeby leaves of a Merlot vineyard.

Mechanisms of sexual reproduction are well analysed and the implications for the development of a population or whole species may be easily assessed. Working on asexual reproduction, one may sometimes feel displaced into times before Mendelian tenets, no solid rules exist for orientation, except the obsolete definition of clones, consisting of genetically identical individuals - but this has now been negated several times (see Lushai & Loxdale 2002 for a review). Using the example of grape phylloxera, characteristics of asexual eucaryotes were illustrated in this thesis, focussing on the presence of intraclonal genetic variation. Intraclonal variation was first determined in studies working on procaryotes, untill it became obvious that this phenomenon also existed for higher organisms, namely plants, rotifers, nematodes, arthropods and vertebrates (Avise *et al.* 1992, Lynch 1984).

But, asexual reproduction has also important applied aspects. Many agricultural pests, and aphids for example, reproduce asexually. Agricultural pest do not live under natural conditions, in which the host plant, natural enemies, competitors and abiotic factors all represent variable factors to which the insect biotype needs to react. Agricultural host plants are very homogenous, especially in the case of clonal *Vitis* species. There are fewer interacting compounds compared to natural environments and we still do not know how asexual organisms may comport here. The relevance of asexual reproduction will certainly grow in the coming years and important mechanisms acting among asexually reproducing pests are expected to be clarified, in order to keep up with the development of adequate plant protection systems. The usefullness of asexually reproducing grape phylloxera as a model organism thus cannot be denied. For viticultural sciences it is important to perceive that the former and well-known life cycle of grape phylloxera is not up-to-date anymore. By now, a range of life cycle variations of this insect have been identified and described. Despite the asexual reproductive mode, this insect holds the potential to rapidly develop new and aggressive biotype, due to the high genetic variation and an enormous reproduction rate. This potential should not be underestimated.

Here, the application of adequate molecular methods certainly is a magnificent mean to answer remaining questions of this insect pest and the complex interaction with its host plant. Furthermore, viticulture will need to react to these changes by developing new strategies of plant protection. No insecticides exist, which control phylloxera infestations efficiently. The development of biological plant protection products based on the insights gained on bacterial interactions with grape phylloxera (Kirchmair *et al.* 2004) might be an alternative strategy. Meanwhile, Australian and Californian viticulture has suggested new practices, as

the monitoring of grape phylloxera spread using arial imaging techniques (Johnson *et al.* 1996, Frazier *et al.* 2004), which may be an interesting complementation to strict sanitary programs for phylloxera damage containment in large viticultural areas. However, the only long-term solution to the phylloxera problem remains the continious development of resistant rootstocks. More information on the genetic mechanisms of interaction between the insect and its host are essential, in order to compile as much „genetic options“ as possible for the progression in rootstock breeding.

9 Summary

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch; Homoptera: Phylloxeridae) is an economical important insect pest of grapevine (*Vitis* spp.) worldwide. The insect was introduced with contaminated plant material from North America in the 1850s and spread rapidly across all European viticultural regions. Grape phylloxera induce galls by feeding on leaves and roots of many grape species, which stresses the plant metabolism and may cause secondary infections especially on the roots of the host. In the 19th century, nearly three-forths of the ungrafted and highly susceptible European grape species were destroyed by the insect pest. European viticulture did not recover until the development of grafting, combining European *Vitis vinifera* varieties with resistant rootstocks, bred from American *Vitis* species.

Grape phylloxera is still present in viticulture. Today, grape phylloxera populations mainly persist in abandoned vineyards and rootstock nurseries. Moreover, the attack of some rootstocks, which had so far been classed resistant to grape phylloxera, was reported from different regions in the past 15 years (Boubals 1994, Granett *et al.* 1985, Kocsis *et al.* 2002). Grape phylloxera populations seem to be variable in terms of genotypic composition and host adaptability. The lifecycle described by Fitch (1854) and others in the 19^{th} century does not seem to match actual conditions anymore.

This thesis aimed at redefining the genetic structure of European grape phylloxera populations by employing genetic markers. It was shown, that the insect has turned away from its classical holocycle and now mainly reproduces asexually, as already demonstrated for Australian grape phylloxera populations (Corrie *et al.* 2002). Despite asexual reproduction, all examined populations revealed a high grade of genotypic diversity. The identified genotypes were specific for each habitat, reflecting the isolated structure of abandoned vineyards and rootstock nurseries as the main habitats of the insect pest. Within habitats, however, a rapid clonal turnover was observed within one summer season, due to an active „microscale“ migration of first instars.

The reports on the emergence of new and more aggressive strains raised the question, how a population composed of asexually reproducing organisms would change and adapt to such an extent. Using a multilocus marker system, eight single founder lineages were genetically monitored over at least 15 generations. All lineages revealed a high grade of intraclonal variation. Sequencing of polymorphic fragments showed, that the genetic variation was not due to contaminating plant or bacterial DNA, but was due to variation within the insect genome. Furthermore, mutations occured already in early generations and were not observed to accumulate in later generations. Mutations were rather generated constantly and only few mutation specific markers were identified to be stable over all following generations. The here documentated genetic variation reveals the great adaptational potential of this insect pest.

The adaptability of single founder lineages was further assessed by measuring physiolog-

ical parameters in single isolation chambers in the greenhouse. Parameters as the number of surviving individuals per generation, the number of eggs or the number of ovarioles per generation exposed differences in performance among the lineages and also within the lineages a high grade of intraclonal variation. A few single founder lineages in the experiment revealed particular adaptation to the new host plant chosen. Additionally, samples of single founder lineages were analysed using the previously employed multilocus marker system. However, a direct correlation of specific markers and particularly adapted individuals or lineages was not possible in this assay. Two markers, though, were observed to occure in several lineages which performed well on the new host plant. These markers may be a first step to the development of adaptation-related markers and need to be tested on further populations and host plants.

When analysing intraclonal variation, the question of putative contaminating factors within the system arises. Symbiotic bacteria occuring in nearly all aphid species certainly are the first to be suspected as a source of genetic variation among single individuals tested. Endosymbiotic bacteria, as *Buchnera aphidicola* in other aphid species, influencing nutritional condition and fitness of the insect population, were not identified in *D. vitifoliae*. A bacterium, closely related to *Pantoea agglomerans*, however, was identified in several grape phylloxera populations, using universal 16S rDNA primers and later specifically developed markers, which were also employed for *in situ* hybridization. The bacterium was localized in the salivary pump of *D. vitifoliae*. PCR analysis of *in vitro* reared populations revealed that the bacterium is present in root- and leaf-feeding parthenogenetic populations of grape phylloxera and, moreover, seems to be transmitted from generation to generation. In other insect species, this bacterium has been demonstrated to produce antifungal and antibacterial substances (Dillon & Charnley 1995, Dillon *et al.* 2000), which were also found in first *in vitro* tests with grape phylloxera associated bacteria. The exact function of *P. agglomerans* in *D. vitifoliae* is still to be defined. The insect may, however, benefit from the antagonistic potential of these bacteria. *P. agglomerans* may be a further participant in the certainly complex interaction of grape phylloxera and grapevine.

This thesis represents a broad approach to elucidate the development of grape phylloxera populations in Europe. Using new molecular marker systems, it has become possible to gain more information on the genetic structure of the insect and its adaptational potential. The predominant clonal reproduction mode of the insect confronts grapevine breeders and pest management with the task to continously develop new resistant rootstocks and to keep up with new pest management systems.

10 Zusammenfassung

Die Reblaus (*Daktulosphaira vitifoliae* Fitch; Homoptera: Phylloxeridae) ist ein wirtschaftlich bedeutender Schädling in allen weltweiten Weinbauregionen. Das aus Nordamerika stammende Insekt wurde Mitte des 19. Jahrhunderts nach Europa verschleppt und breitete sich rasant in allen europäischen Weinbaugebieten aus. Durch die Saugtätigkeit an Blättern und Wurzeln der Rebe (*Vitis* spp.) werden Gallen induziert, welche den Stoffwechsel der Rebe stark beanspruchen und vor allem an den Wurzeln die Gefahr von Sekundärinfektionen mit sich bringen. Dreiviertel der damals ungepropften und damit hochanfälligen europäischen Reben wurden durch den Schädling vernichtet. Erst die Einführung von Pfropfreben - die Verbindung europäischer Edelreiser mit amerikanischen, wurzelresistenten Unterlagen - machte es möglich, die Rebflächen sicher wieder aufzureben.

Die Reblaus ist trotzdem auch heute noch im Weinbau präsent. Sie vermehrt sich insbesondere an ausgetriebenen Unterlagen (sogenannten Drieschen) und in Unterlagsschnittgärten. Ausserdem wurde in den letzten Jahrzehnten häufiger vom Befall bisher als resistent eingestufter Unterlagen neuen und aggressiveren Reblausstämmen berichtet (Boubals 1994, Granett *et al*. 1985, Kocsis *et al*. 2002). Reblauspopulationen scheinen in Hinsicht auf ihre Wirtsanpassungsfähigkeit und ihre genetische Zusammensetzung sehr variabel zu sein. Der im 19. Jahrhundert beschriebene Lebenszyklus entspricht sicher nicht mehr den aktuellen Gegebenheiten.

Ein wichtiges Ziel dieser Arbeit war daher, die genetische Struktur europäischer Reblauspopulationen mit Hilfe genetischer Marker neu zu definieren. Es zeigte sich, wie auch schon in anderen Weinbaugebieten, z.B. Australien nachgewiesen (Corrie *et al*. 2002), dass sich die Reblaus vorwiegend asexuell vermehrt und nicht mehr den klassischen holozyklischen Lebenszyklus aufweist. Trotz der klonalen Vermehrungsweise konnte in den untersuchten Populationen eine sehr grosse genotypische Diversität nachgewiesen werden. Die identifizierten Genotypen waren für jedes untersuchte Habitat spezifisch und spiegelten die isolierte Habitatsttruktur und die vorwiegende Beschränkung des Schädlings auf sogenannte Drieschen und Unterlagsschnittgärten wider. Innerhalb der Habitate konnte jedoch über eine Vegetationsperiode hinweg ein reger Wechsel dominierender Genotypen beobachtet werden, ein Hinweis auf aktive Migration innerhalb des Habitats.

Die Verbreitung neuer und agressiverer Stämme warf die Frage auf, wie sich eine aus klonalen Organismen bestehende Population derart schnell verändern und anpassen kann. Mit Hilfe eines Multilocus-Markersystems wurden acht klonale Linien über mindestens 15 Generationen genetisch analysiert. Alle Linien zeigten einen hohen Grad an intraklonaler genetischer Variation. Die Sequenzierungen polymorpher Fragmente zeigten, dass die genetischen Unterschiede innerhalb eines Klons nicht von kontaminierender bakterieller oder pflanzlicher DNS herrührten, sondern insekteneigene, genetische Variationen darstellten. Weiterhin wurde festgestellt, dass sich die bereits in frühen Generationen auftretenden Mutationen zum

grossen Teil nicht bis in spätere Generationen akkumulierten. Vielmehr wurden kontinuierlich neue Mutationen generiert und nur einige wenige etablierten sich fest in den folgenden Generationen. Die hier zum ersten Mal auf diese Weise dokumentierte genetische Variabilität klonaler Reblauslinien macht das Adaptationspotential dieses Schädlings deutlich.

Die Anpassungsfähigkeit einzelner klonaler Linien wurde zusätzlich mit Hilfe von einfach messbaren physiologischen Parametern im Gewächshaus ermittelt. Dabei wurden Single-Founder-Linien auf neue Wirtspflanzen in Isolationskäfigen vermehrt und beobachet. Auch an Parametern, wie der Anzahl überlebender Individuen pro Generation, der Anzahl Eier oder auch der Anzahl Ovariolen konnte eine intraklonale Variation bei den Linien nachgewiesen werden. Einige Linien zeigten eine besonders gute Anpassungsfähigkeit an die verwendete Wirtspflanze. Zusätzlich wurden Stichproben dieser Linien mit dem bereits verwendeten Multilocus-Markersystem analysiert. Eine direkte Korrelation spezifischer Marker mit besonders angepassten Individuen konnte nicht festgestellt werden. Die Identifikation zweier häufig auftretender Marker ist jedoch ein erster Schritt zur Entwicklung adaptationsspezifischer Marker, die dann an weiteren Populationen getestet werden sollen.

Bei der Analyse intraklonaler Variation stellt sich immer die Frage nach möglichen Kontaminationen, die diese verursachen können. Die Vermutung liegt nahe, dass symbiontische Bakterien, wie sie bei fast allen Aphiden vorkommen, eine wichtige Quelle für genetische Variation sein könnten. Endosymbiontische Bakterien, wie *Buchnera aphidicola* bei anderen Blattlausarten, welche direkten Einfluss auf Ernährung und Fitness der Insektenpopulationen haben, konnten bei der Reblaus nicht identifiziert werden. Jedoch wurde ein dem Bakterium *Pantoea agglomerans* nah verwandtes Bakterium in allen untersuchten Reblauspopulationen nachgewiesen. Dies geschah durch Sequenzierung der mit universellen Primern amplifizierten 16S rDNS-Fragmente und später mit Hilfe von spezifischen Markern, die auch zur *in situ* Hybridisierung verwendet wurden. Das Bakterium konnte in der Speichelpumpe von *D. vitifoliae* lokalisiert werden. PCR Analysen von *in vitro* Populationen zeigten, dass das Bakterium nicht nur in Blattpopulationen, sondern auch bei Wurzelläusen vorkommt und von Generation zu Generation übertragen wird. In anderen Insektenarten konnte eine antifungale und antibakterieller Funktion bereits nachgewiesen werden (Dillon & Charnley 1995, Dillon *et al*. 2000). Erste *in vitro* Tests bestätigten dies auch für das hier identifizierte Bakterium. Die genaue Funktion in der Reblaus muss jedoch noch ermittelt werden. Es ist vorstellbar, dass die Reblaus vom antagonistischen Potential dieses Bakteriums profitiert. *P. agglomerans* könnte somit ein weiterer interessanter Mitspieler in der komplexen Rebe-Reblaus Interaktion sein.

Die vorliegende Arbeit stellt einen breit gefächerten Ansatz zur Aufklärung des Verhaltens klonaler Reblauspopulationen in Europa dar. Mit Hilfe neuer molekularer Methoden ist es möglich geworden, neue Informationen zur genetischen Struktur dieses Schadinsekts zu gewinnen und seine Anpassungsfähigkeit zu beobachten. Die klonale Vermehrungsweise stellt den Rebschutz vor die Aufgabe, das Verhalten dieses sich rasant entwickelnden Schäd-

lings genau zu beobachten und die Entwicklung resistenter Unterlagen und neuer Pflanzenschutzmassnahmen voranzutreiben.

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Tätigkeiten

09/1998-02/1999	Praktikum "Microorganisms and nutrient cycling in desert soils", Dep. of Agronomy and Horticulture, New Mexico State University, Dr. A. Ulery
04/1999-06/1999	landwirtschaftliches Praktikum im Landesweingut Kloster Pforta, Bad Kösen
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05/2002-08/2002	molekularbiologischer Teil der Diplomarbeit, Instituto de Cavanilles de Biodiversidad y Genetica Evolutiva, Universidad Valencia, Spanien, Dr. D. Martinez-Torres

EDV-Kenntnisse

WIN, MAC OS9, OSX - Populationsgenetik, Sequenzanalyse und Bildverarbeitung: Arlequin, Genepop, FSTAT, NTSYS, ClustalX, MEGA3, Axiovision, Photoshop

Sprachkenntnisse

Englisch, Französisch, Spanisch (sehr gut in Wort und Schrift), Finnisch, Schwedisch (Grundkenntnisse)