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Adaptational potential of grape phylloxera (*Daktulosphaira vitifoliae*) clonal lineages

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The performance of 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 intraclonal 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.
Key words: Phylloxeridae, adaptation, abandoned vineyards, 'Teleki 5C', 'Cabernet Sauvignon'

***Das Adaptationspotenzial klonaler Linien der Reblaus* (Daktulosphaira vitifoliae)**. *Die Vitalität von dreißig klonalen Linien der Reblaus wurde über fünf Generationen an 'Teleki 5C' und 'Cabernet Sauvignon' anhand von leicht messbaren Leistungsparametern überprüft. Zusätzlich wurden AFLP-molekulare Fingerprints für eine Teilmenge von sechs klonalen Linien ausgewertet. Klonale Linien entwickelten sich besser an der ursprünglichen Wirtspflanze ('Teleki 5C') als auf der neuen ('Cabernet Sauvignon'). Dieses konnte an der Zahl überlebender klonaler Linien pro Generation, der reproduktionsfähigen adulten Individuen sowie an der Lebensspanne der Generationen und der Zahl der Ovariolen gezeigt werden. Mit genetischen Fingerprints konnte ein hohes Maß an inter- und intraklonaler Variabilität in allen sechs untersuchten Linien gefunden werden. Zwei wahrscheinliche wirtspezifische Marker wurden identifiziert. Genetisch und phänotypisch hoch variable Reblauspopulationen haben das Potenzial für kurzfristige Anpassung an neue Wirtspflanzen und können sich zu neuen aggressiven Biotypen entwickeln. Dieser Umstand muss für zukünftige Pflanzenschutzstrategien im Weinbau in Betracht gezogen werden.*
Schlagwörter: Reblaus, Adaptation, aufgelassene Weingärten (Drieschen), 'Teleki 5C', 'Cabernet Sauvignon'

***Le potentiel d'adaptation des lignes clonales du phylloxéra de la vigne* (Daktulosphaira vitifoliae)**. *La vitalité de trente lignes clonales du phylloxéra de la vigne a été vérifiée pendant cinq générations sur 'Teleki 5C' et 'Cabernet Sauvignon' à l'aide de paramètres de performance facilement mesurables. En outre, les empreintes digitales moléculaires AFLP ont été analysées pour un sous-ensemble de six lignes clonales. Les lignes clonales se sont mieux développées sur la plante-hôte originale ('Teleki 5C') que sur la plante-hôte nouvelle ('Cabernet Sauvignon'). Ceci a pu être démontré à l'aide des lignes clonales survivantes par génération, des individus adultes capables de se reproduire, ainsi qu'à l'aide de la durée de vie des générations et du nombre des ovarioles. Les empreintes digitales génétiques ont permis de trouver une variabilité inter- et intraclonale élevée dans l'ensemble des six lignes examinées. Deux*

marqueurs probablement spécifiques aux hôtes ont été identifiés. Les populations de phylloxéra hautement variables du point de vue génétique et phénotypique possèdent le potentiel de s'adapter rapidement aux nouvelles plantes-hôtes et peuvent se développer en nouveaux biotypes agressifs. Il faut tenir compte de cette circonstance dans le cadre du développement de nouvelles stratégies phytosanitaires dans la viticulture.
Mots clés : phylloxéra, adaptation, vignobles en friche, 'Teleki 5C', 'Cabernet Sauvignon'

Over the last years, an increasing number of fallow vineyards have developed 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 commercial vineyards allowing migration of phylloxera populations. Although *Vitis vinifera*-based hosts are described to be not susceptible to leaf-feeding phylloxera, galls can be easily 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.

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 (Askani and Beiderbeck, 1991; Grzegorczyk and Walker, 1998; Kocsis et al., 2002). The work of Forneck et al. (2001a) and 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 and Forneck, 2006a) 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?
(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 and 2001b; Vorwerk

and FORNECK, 2006a).
Inoculation was effected by placing 25 to 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 *V. berlandieri* x *V. riparia* cv. 'Teleki 5C' (T5C) and of *V. vinifera* cv. 'Cabernet Sauvignon' (CS). 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 and CS.
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 to 260 mg/l nitrogen, 180 to 280 mg/l phosphate, 200 to 350 mg/l potassium oxide, 80 to 150 mg/l magnesium oxide, pH ($CaCl_2$): 5 to 6). One pre-rooted cutting was planted into each 600 ml of soil soaked with 200 ml of water. Isolation chambers were prepared two weeks prior to inoculation. No fertilizer was applied to eliminate interacting effects on the phylloxera.

Phylloxera performance (Experimental design)

Performance of thirty single founder lineages on two treatments, T5C and CS, was observed over five generations (G_1 to 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 reoccuring 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 to 490 nm, FT 510, LP 520).

Table 1: Primer sequences employed in the study (* indicates cy5-labeled primers)

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

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 was 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 and FORNECK (2006b). Four primer combinations using 3'-cy5'-labeled primers were used: E10*/M8, E14*/M8, E16*/M8, E21*/M17 (Table 1). 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*.

Table 2: Comparison of performance parameters for the host plants T5C and CS

Generation	Surviving lineages		Reproducing adults		Number of ovarioles	
	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 sample numbers parameters could not be clearly measured in these generations

An original host lineage, collected from T5C, was considered as adapted to a new host (CS) when its performance was not significantly weaker than comparable lineages on the original host.

Performance on host plant

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 2). The generation time was significantly longer for lineages on CS (20.6 d +/-1.8) compared to T5C (19.1 d +/-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. 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 2).

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. Single CS lineages, however, 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 3). 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.

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, number 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

Table 3: Descriptive Analysis of AFLP Screening of the individual clonal lineages tested

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

* less than 5,00% missing data

** markers in bold type indicate host specifity

host plant with respect to the aphid's performance could be confirmed.
Most of the clonal lineages performed better on their hosts of origin (T5C) than they did on the alternate host (CS) 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 (Granett et al., 1985; Hawthorne and Via, 1994; Forneck et al., 2001a; 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 host CS from G_2 on (data not presented). Changes in body size in relation to host plant factors were also detected by Wool and 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 showed at least a certain short-term adaptation to their new host, but 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 (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 to compare 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 and Forneck, 2006a), 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 and Forneck, 2006a), 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 (Forneck et al., 2001b). 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 and Forneck, 2006b).

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. Further 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 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.

Consequently there must have 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 and Forneck, 2006b) or for galled *Vitis* roots and *Metarrhizium* (Huber et al., 2006).

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), these lineages, however, 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 and Forneck (2006b) 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 (Di Pietro and 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 commercial viticulture, growing monocultural plantings for 20 to 30 years, 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 a new invasion.

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