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Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA

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Summary

Grapevines, alternative host plants, and vectors from different viticultural areas of Germany were surveyed for the presence of stolbur-group phytoplasmas that are associated with grapevine yellows. Isolates from these field samples and periwinkle isolates of stolbur were characterised by RFLP-analysis of non-ribosomal DNA fragments using a combination of 6 polymorphic primer-enzyme combinations. Three of the 5 different restriction profiles that could be distinguished were found in grapevine and other field samples of plants and insects. A previously unknown isolate was detected in grapevine, vectors and the newly identified herbaceous host *Calystegia sepium*. The further analysis of the phylogenetic relationship between the 5 RFLP groups revealed a close relationship between two of the isolates associated with grapevine yellows which were also found in the two Convolvulaceae *Convolvulus arvensis* and *Calystegia sepium*. The third isolate which was detected in grapevine and stinging nettle *Urtica dioica* is more closely related to a periwinkle isolate obtained from *Lavandula officinalis* than to the other two grapevine yellows isolates. Based on the data of the field survey a specific association of the three isolates to different alternative host plants is discussed.

K e y w o r d s : Grapevine yellows, vector, phytoplasma, epidemiology.

Introduction

Phytoplasmas associated with grapevine yellows (GY) belong to different groups (Boudon-Padieu 2003). Bois noir (BN), the most widespread GY in Europe and the Mediterranean is caused by pathogens of the stolbur group. It is called 'Vergilbungskrankheit' (VK) in Germany. Phytoplasmas of this group infect a wide range of wild and cultivated herbaceous plants beside grapevine (Marcone *et al*. 1997; Schneider *et al*. 1997 a). Known vectors of stolbur group phytoplasmas are the Cixiid planthoppers *Hyalesthes obsoletus* Signoret 1867 the vector of VK and BN (Maixner *et al*. 1995; Sforza *et al*. 1998), and *Pentastiridius beiieri* Wagner 1970 that transmits stolbur to sugar beet (Gatineau *et al*. 2001). The phytoplasmas are maintained by a cyclic change between herbaceous plants and the planthopper vector. Occasional erroneous feeding of the planthopper results in the inoculation of grapevine and subsequent development of GY in this plant. Principal herbaceous host plants of *H. obsoletus* in vineyards are the bindweeds *Convolvulus arvensis* L. and *Calystegia sepium* L., perennial species of *Ranunculus*, stinging nettle *Urtica dioica* L. and hoary cress *Cardaria draba* (L.) Desv. (Sforza *et al*. 1998; Vidano *et al*. 1988; Weber and Maixner 1998), but only the bindweeds and nettle are consistently infected by the stolbur phytoplasma in Germany.

The differentiation and classification of culturable mollicutes is based on both phenotypic and genotypic characteristics (ICSB Subcommittee on the Taxonomy of Mollicutes 1995). Classification of phytoplasmas was first accomplished by RFLP analysis of the conserved 16S rRNA gene (Lee *et al.* 1993; Schneider *et al*. 1993). The monophyletic clade of the phytoplasmas could be differentiated into 14 major 16S rDNA groups (16 Sr I-XIV) and 32 subgroups (Lee *et al*. 1998). The stolbur group was divided into two subgroups (A and B), typified by Serbian stolbur from pepper (STOL) and Australian grapevine yellows (*Candidatus phytoplasma australiense,* Davis *et al*. 1996), respectively (Lee *et al*. 1993, 1998). Further phylogenetic analysis by direct sequence comparison of the 16S rDNA gene led to the subdivision of the phytoplasma clade into 20 subclades, several of them including more than one putative taxonomic unit (Seemüller *et al*. 1998).

Genetic variability based on RFLP analysis is low within the stolbur subgroup, compared to other groups like aster-yellows (Marcone *et al*. 2000). However, considerable differences in genome sizes have been reported (Marcone *et al*. 1999; Schneider *et al*. 1997 a). Reinert and Maixner (2000) described two different RFLP-types associated with grapevine yellows in Germany. Because of the low variability of ribosomal sequences within the stolbur group we included additional, non-ribosomal DNA-fragments (Schneider and Seemüller 1996; Daire *et al*. 1997) in the study. Due to their higher sequence variability they appeared to be more suitable for the differentiation and classification of the closely related phytoplasmas.

The aim of this study was to further characterise stolbur phytoplasma isolates associated with grapevine yellows in German viticulture by RFLP-analysis and to investigate the association of different isolates with several natural host plant species and with field populations of the vectoring planthopper. The data obtained lead to the assumption that different natural cycles of the phytoplasma could exist in the field which are of varying significance for the epidemiology of VK.

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Material and Methods

Plant and insect samples: Different phytoplasma isolates of the stolbur-group from periwinkle and natural host plants were examined (Tab. 1). A periwinkle isolate of aster yellows (AV 2192, SCHNEIDER *et al*. 1993) was included as an outgroup.

Samples from naturally infected plants were taken from grapevine and the alternative host plants *Convolvulus arvensis*, *Urtica dioica* and *Calystegia sepium* in several viticultural areas in Germany (Tab. 2). DNA obtained from infected *Urtica dioica* from France was provided by E. BOUDON-PADIEU (INRA, Dijon, France).

Adult *Hyalesthes obsoletus* caught alive from herbaceous hosts in various German viticultural areas were included in this work. A total number of 2083 *Hyalesthes* was collected from the predominant host plants of all collection sites (Tab. 3). At sampling sites with two or three host spe-

Table 1

Periwinkle isolates of phytoplasmas in this study

Phytoplasma	Geographic origin	Strain/ isolate	Taxonomic group	Reference	Genome size (kb)*
Lavender decline	France	DEPL	Stolbur	COUSIN *et al*. 1970	990
Grapevine yellows	Italy	CA-1	Stolbur	CREDI 1993	1070
Grapevine yellows	Italy	CH-1	Stolbur	CREDI 1993	945
Grapevine yellows	Italy	SA-1	Stolbur	CREDI 1993	1020
Grapevine yellows	Italy	SA-2	Stolbur	CREDI 1993	1080
Grapevine yellows	Germany	GGY	Stolbur	MARCONE *et al*. 1996	860
Grapevine yellows	Germany	VK 1925	Stolbur	MAIXNER unpubl.	
Grapevine yellows	Spain	17	Stolbur	BATTLE and LAVINA unpubl.	
From sour cherry	France	MOL	Stolbur	SCHNEIDER *et al*. 1993	1220
Stolbur of pepper	Serbia	STOL	Stolbur	SEEMÜLLER *et al*. 1998	900
Tomato stolbur	France	STOLF	Stolbur	SCHNEIDER *et al*. 1993	1220
Aster yellows	Germany	AV 2192	Aster yellows	SCHNEIDER *et al*. 1993	1125

* Genome sizes published by MARCONE *et al*. (1999).

Table 2

Species and origins of samples of herbaceous alternative host plants of VK-phytoplasma collected in the field

Plant species	n tested	n infected	Origin (viticultural area)
Convolvulus arvensis	164	16	Nahe, Baden, Mosel
Urtica dioica	43	3	France, Mosel, Middle Rhine
Calystegia sepium	158	3	Mosel

Table 3

Number, geographic origin, and host plants of *H. obsoletus* specimen collected in the field

n tested	n infected	% infected	Origin	Viticulrural area	Host plant
170	63	37	Zell	Mosel	*C. arvensis*
141	44	31	Graach	Mosel	*C. arvensis*
62	19	31	Kröv	Mosel	*C. arvensis*
137	78	57	Maring	Mosel	*C. arvensis*
45	18	40	Reil	Mosel	*C. arvensis*
260	93	36	Bernkastel-Kues	Mosel	*C. arvensis*
285	81	28	Ungstein	Palatinate	*U. dioica*
216	116	54	Bacharach	Middle-Rhine	*U. dioica*
					U. dioica
553	130	24	Lehmen	Mosel	*C. arvensis*
					C. sepium
214	3	1	Kesten	Mosel	*Ranunculus* spp.

cies growing together, the planthoppers were caught separately from each host-plant.

DNA extraction and amplification: Total DNA from insects and host plants except of *Urtica dioica* was isolated using a modified CTAB procedure (Maixner *et al*. 1995). Detection of phytoplasma in nettle was difficult. It required a special DNA isolation procedure to obtain sufficient amounts of phytoplasma DNA. Leaf midribs (700 mg) were ground in liquid nitrogen using mortar and pestle. A total volume of 7 ml ice-cold grinding buffer was added (100 mM K_2HPO_4, 30 mM KH_2PO_4, 10 % sucrose, 0.15 % BSA, 2 % PVP-10, 25 mM ascorbic acid, pH was adjusted to 7.6). The homogenate was transferred into a 10 ml Oak Ridge Centrifuge Tube (Nalgene; Rochester, USA) and centrifuged for 5 min at 5.400 g at 4 °C. The clear supernatant was transferred into 2 ml reaction tubes and centrifuged for 30 min at 22.000 g at 4 °C. DNA from the enriched pellet was resuspended with CTAB buffer and processed as described above.

DNA was amplified from both ribosomal and non-ribosomal sequences. Polymerase chain reaction mixtures of 20 µl consisted of approximately 100 ng of template DNA, 5 U Dynazyme Polymerase (Finnzymes; Espoo, Finland), 1x Dynazyme reaction buffer (Finnzymes), concentrations of 0.5 µM of each primer and 200 µM of each dNTP (Amersham Bioscience, Freiburg, Germany). PCR was carried out in an Eppendorf Mastercycler Gradient (Eppendorf; Hamburg, Germany) with 34 cycles. Tab. 4 shows primers, primersequences and PCR conditions.

RFLP-analysis of PCR-products: Reaction products obtained by PCR were digested by a series of restriction enzymes. TufAY PCR products were digested by the enzymes *Mse*I, *Hpa*II, *Dra*I (MBI, Lithuania), *Rsa*I, *Hind*III, *Hinf*I (Amersham Bioscience; Freiburg, Germany) and *Taq*I (Sigma-Aldrich; Deisenhofen, Germany). The same set of restriction endonucleases except *Taq*I was used with the ribosomal P1/P7 PCR fragment. The STOL4 fragment was digested with *Dra*I, *Ssp*I (MBI, Lithuania) and *Mbo*I (Amersham Bioscience; Freiburg, Germany), the fragment STOL11 with *Dra*I, *Mse*I and *Nde*II (Reinert 1999) and the fragment M1/P8 with *Hpa*II.

Volumes of 20 µl of PCR product were digested separately with 7 U of each restriction enzyme for at least 2 h at 37 °C (*Taq*I and *Mse*I at 65 °C) in buffer supplied with the enzymes.

Fragments were separated electrophoretically in 5 % polyacrylamidgels (PAGE), stained with ethidiumbromid, visualized under UV light and photographed for documentation. Molecular weights were determined using a 1 kb DNA ladder (Invitrogen, Karlruhe, Germany) and the Gel-Pro express software Version 4.0 (Media Cybernetics, Silver Spring, USA).

Data analysis: Each band in each sample was scored as present or absent. These data were used to determine variations within accessions. The relatedness between phytoplasma strains was calculated according to Lynch and Milligan (1994) using the Aflp-Surv software (Vekemans *et al*. 2002). A phylogenetic tree was established using the RFLP data. Dendrograms were produced by the program Mvsp Version 3.13g (http://www.kovcomp.com) using the relatedness according to Lynch and Milligan (1994). UPGMA (unweighted pair group averages) was used as clustering method and the euclidian distance as a distance measure.

Relative support for the internal nodes of the trees was estimated by bootstrap analysis (Felsenstein 1985) consisting of 10000 pseudoreplicates in Phylip Version 3b (Felsenstein 2002) using the "extended majority rule". The periwinkle isolate AV 2192 was used as an outgroup. The data of the genetic relatedness according to Lynch and

Table 4

Primer sequences and PCR conditions

Primer	Sequence 5´3´	initial denaturation	denaturation	annealing	extension	final extension
f tufAY	GCTAAAAGTAGAGCTTATGA[1]	94 °C	94 °C	53 °C	72 °C	72 °C
r tufAY	CGTTGTCACCTGGCATTACC[1]	4 min	30 s	30 s	1 min	5 min
fStol4	TTTAGCGATATTGGGAGAA[2]	94 °C	94 °C	58 °C	72 °C	72 °C
rStol4	ATCCTTGAATTCTTTGACG[2]	4 min	30 s	1 min	45 s	30 s
fP1	AGAGTTTGATCCTGGCTCAGG[3]	94 °C	94 °C	45 °C	72 °C	72 °C
rP7	CGTCCTTCATCGGCTCTT[4]	1 min	1 min	1 min	1.30 min	30 s
Stol11 F2	TATTTTCCTAAAATTGATTGGC[5]	94 °C	94 °C	55 °C	72 °C	72 °C
Stol11 R1	TGTTTTTGCACCGTTAAAGS[5]	2 min	40 s	40 s	1.10 min	2 min
Stol11 F3	ACGAGTTTTGATTATGTTCAC[5]	94 °C	94 °C	55 °C	72 °C	72 °C
Stol11 R2	GATGAATGATAACTTCAACTG[5]	2 min	40 s	40 s	1.10 min	2 min
M1	ACTTATTTTCACAACAACGG[6]			Touch-		
P8	TGTCTAATTCTCCTTCAGGG[6]	94 °C	94°C	down	72°C	72°C
		2 min	1.30 min	69 °C-	45 s	4 min
				55 °C		

[1] Schneider *et al.* 1997 b; [2] Daire *et al*. 1997; [3] Deng and Hiruki 1991; [4] Smart *et al*. 1996.
[5] Clair *et al*. 2003 and [6] Marzachi *et al*. 2000.

MILLIGAN (1994) were processed additionally using a principal component analysis (PCA) and the relative positions of the different types were shown in a three dimensional plot of the major components.

Results

Fragments of expected size were amplified with all primer sets from all stolbur isolates except of MOL that could not be amplified with primers Stol4 f/r (DAIRE *et al*. 1997).

Restriction profiles obtained from the ribosomal fragment P1/P7 and the non-ribosomal fragments Stol11 and M1/P8 showed no polymorphism, while the primer/emzyme-combinations tufAY/*Taq*I, tufAY/*Hpa*II, tufAY/*Hind*III, tufAY/*Hinf*I, Stol4/*Dra*I and Stol4/*Ssp*I revealed a genetic variability within the analysed isolates (Tab. 5).

The analysis of the restriction patterns of the 6 polymorphic primer/enzyme-combinations yielded 33 polymorphic RFLP-markers (Tab. 6).

It was possible to amplify a specific DNA fragment with primer Stol4 f/r from all strains except of AV 2192 and MOL which appears in that respect distinct from the other strains in the Stolbur subgroup. Undigested PCR fragments of Stol4 showed a small difference in size. Three different fragments could be distinguished with sizes of approximately 2000 bp (GGY), 2060 bp (DEPL), and 2100 bp (VK1925, data not shown).

Digestion of the Stol4 fragments with *Dra*I (Fig. 1) and *Ssp*I led to three different restriction profiles. Two of them (Type A and B) where detected in field samples and one in the periwinkle isolate DEPL (Tab. 7). Type A which had only been known from the Palatinate area beside periwinkle isolates was now also detected in the viticultural areas of Mosel, Middle Rhine, Baden, Württemberg, Nahe and Bergstraße. Type B is the most widespread; it is present in all of the 10 viticultural areas that were analysed so far, while type C was slightly different from A and unique for the isolate DEPL (Fig. 1).

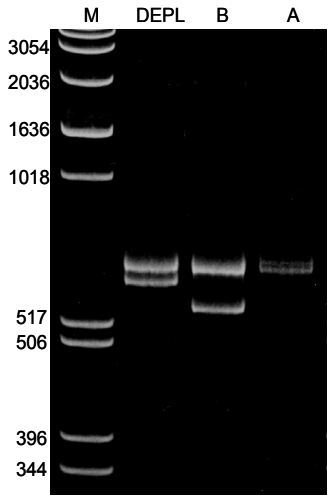

Fig. 1: *Dra*I restriction profiles of a non-ribosomal DNA fragment amplified by PCR using primers f/rStol4. M: Molecular weight marker; DEPL: Lavender decline; B: VK-isolate from grapevine, RFLP-type B; A: VK-isolate from grapevine, RFLP-type A.

Table 5

Primer/restriction enzyme combinations used for RFLP analyses and observed polymorphisms

Primer	Enzyme	Polymorphism
f/r tufAY	*Rsa*I	-
	*Mse*I	-
	*Taq*I	+
	*Hpa*II	+
	*Dra*I	-
	*Hind*III	+
	*Hinf*I	+
f/r STOL4	*Dra*I	+
	*Ssp*I	+
	Mbo	-
f/r Stol11	*Dra*I	-
	*Mse*I	-
	*Nde*II	-
fM1/rP8	*Hpa*II	-
fP1/rP7	*Rsa*II	-
	*Mse*I	-
	*Hpa*II	-
	*Hind*III	-
	*Hinf*I	-
	*Alu*I	-

Primers tufAY f/r allowed the specific amplification of a 940 bp fragment from phytoplasmas of the aster yellows and stolbur groups (SCHNEIDER *et al*. 1997 b). Digestion with *Hpa*II resulted in a set of eight polymorphic fragments (Tab. 6, Fig. 2). This allowed the differentiation of stolbur and aster yellows isolates and led to three different restriction profiles (Type a, b, c) of stolbur that were detected in grapevine, herbaceous plants and the vectors as well. Two of them were also found in periwinkle isolates (Type a and b).

The primer/enzyme-combinations tufAY/*Hinf*I, tufAY/*Hind*III, and tufAY/*Taq*I showed no polymorphism within the stolbur-isolates included in this work, but they allowed a differentiation of the Aster yellows isolate. In grapevine as well as in the vector *H. obsoletus*, we detected all three RFLP types (Tab. 6). Type a was only detected in *Urtica dioica*

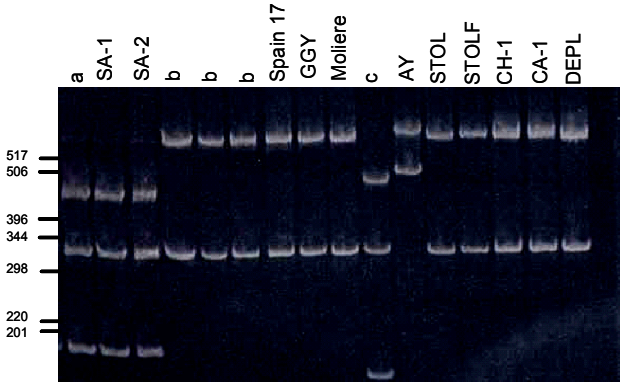

Fig. 2: Polyacrylamide gel showing restriction profiles achieved by digestion of a non- ribosomal phytoplasma DNA fragment amplified by PCR using primers f/r tufAY with *Hpa*II. a, b, and c are field samples (infected *Hyalesthes obsoletus*). For abbreviations of phytoplasma isolates see Tab. 1.

Table 6

Results of RFLP-analyses of different DNA-fragments obtained from field and periwinkle isolates of stolbur phytoplasmas and an aster yellows isolate. Presence/absence of a fragment is indicated by 1/0

		Represenative field samples										Periwinkle isolates								
Primer / enzyme	Fragment size (bp)	Grapevine			*Hyalesthes obsoleteus*			*C. sepium*		*C. arvensis*	*U. dioica*	SA-1, SA-2	VK 1925	CA-1, CH-1	GGY, Spain 17	STOLF	STOL	MOL	DEPL	AV 2192
		1	2	3	1	2	3	1	2	1	1									
tufAY / *Hpa*II	525	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
tufAY / *Hpa*II	520	0	1	0	0	1	0	1	0	1	0	0	0	1	1	1	1	1	1	0
tufAY / *Hpa*II	450	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
tufAY / *Hpa*II	440	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
tufAY / *Hpa*II	410	1	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
tufAY / *Hpa*II	300	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
tufAY / *Hpa*II	170	1	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
tufAY / *Hpa*II	150	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
tufAY / *Hinf*I	1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
tufAY / *Hinf*I	500	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
tufAY / *Hinf*I	490	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
tufAY / *Hind*III	750	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
tufAY / *Hind*III	410	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
tufAY / *Hind*III	405	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
tufAY / *Hind*III	400	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
tufAY / *Hind*III	180	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
tufAY / *Taq*I	530	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
tufAY / *Taq*I	500	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
tufAY / *Taq*I	450	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Stol4 / *Dra*I	710	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0
Stol4 / *Dra*I	670	1	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
Stol4 / *Dra*I	630	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Stol4 / *Dra*I	570	0	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0
Stol4 / *Ssp*I	730	1	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0
Stol4 / *Ssp*I	650	0	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0
Stol4 / *Ssp*I	420	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0
Stol4 / *Ssp*I	380	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Stol4 / *Ssp*I	370	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
Stol4 / *Ssp*I	290	1	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
Stol4 / *Ssp*I	275	0	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0
Stol4 / *Ssp*I	240	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Stol4 / *Ssp*I	170	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0
Stol4 / *Ssp*I	125	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0

and type b only in *Convolvulus arvensis*. *Calystegia sepium,* on the other hand, hosted two different types (b, c). The isolate DEPL was identical to type b. The differentiation of type b and type c with Stol4/*Dra*I was not possible.

As a result of the RFLP-analysis of the Stol4 and tufAY fragments three types of stolbur phytoplasma that are associated with Vergilbungskrankheit could be distinguished. Two additional types, represented by the periwinkle isolates DEPL and MOL could not be detected in our field samples (Tab. 7). VK-Type I (Stol4-A, tufAY-a) was detected in *Vitis vinifera*, *H. obsoletus, Urtica dioica* and the periwinkle isolates SA-1 and SA-2 from grapevine in Italy. This type is more widely distributed in German viticultural areas than we thought before. It was detected in grapevine and *H. obsoletus* from most of the viticultural areas affected with Bois noir. VK-Type II (Stol4-B, tufAY-b) is the most widespread. It was found in grapevine, *H. obsoletus* and the two *Convolvulaceae C. arvensis* and *C. sepium* (Tab. 8). VK-Type III (Stol4-B, tufAY-c) was identified exclusively in *V. vinifera*, *C. sepium* and *H. obsoletus* from the Mosel area.

Table 7

Differentiation of three types of VK-phytoplasma based on RFLP-analysis of the non-ribosomal DNA fragments Stol4 and TufAY obtained by PCR amplification of phytoplasma DNA from grapevine, periwinkle, natural herbaceous host plants and the vector *H. obsoletus*

VK-Type	RFLP profiles Stol4 / *Dra*I	tufAY / *Hpa*II	Source of phytoplasma	Geographic distribution in Germany (viticultural areas)
I	A	a	*Vitis vinifera* *Urtica dioica* (Germany, France[1]) *Hyalesthes obsoletus* (collected from *U. dioica* and *C. sepium*) (Periwinkle: SA-1, SA-2, VK1925)	All viticultural areas affected by VK, except Franconia
II	B	b	*Vitis vinifera* *Convolvulus arvensis* *Solanum nigrum* *Prunus spinosa* *Hyalesthes obsoletus* (collected from *C. arvensis* and *C. sepium*) (Periwinkle: CH-1, CA-1, GGY, Stol, StolF, Spain 17)	All viticultural areas affected by VK
III	B	c	*Vitis vinifera* *Calystegia sepium* *Hyalesthes obsoletus* (collected from *C. sepium*)	Mosel
-*	C	b	(Periwinkle: DEPL)	-
-*	-	b	(Periwinkle: MOL)	-

* not present in grapevine.
[1] DNA sample provided by E. Boudon-Padieu, INRA, Dijon, France.

Table 8

Sample size and classification of Phytoplasmas detected in *H. obsoletus* and host plants

Type of sample	PCR-Test (tufAY) tested	positive	VK-Type[1] I	II	III
Calystegia sepium	158	5	0	3	2
H. obsoletus[2]	122	14	1	3	5
Urtica dioica	51	3	3	0	0
H. obsoletus[2]	604	242	199	5	0
Convolvulus arvensis	164	12	0	5	0
H. obsoletus[2]	538	209	0	99	0
Vitis vinifera[3]	79	56	32	19	2

[1] not all positive samples were characterised by RFLP-analysis.
[2] Vectors collected from the corresponding host plant.
[3] symptomatic grapevine.

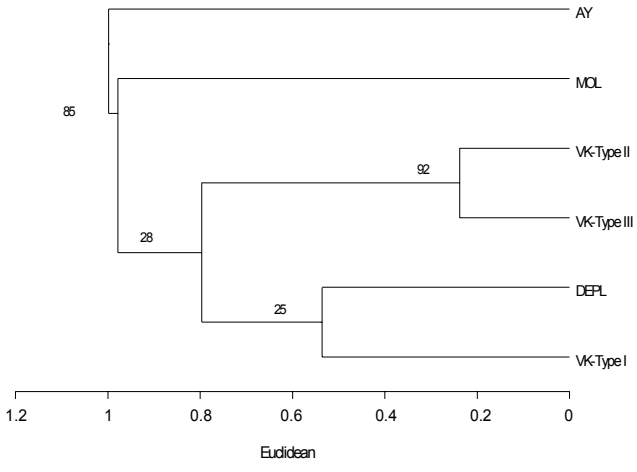

Fig. 3: Phylogenetic tree constructed by UPGMA analysis of RFLP-data based on the genetic relatedness (LYNCH and MILLIGAN 1994). Numbers above branches indicate bootstrap values (%) obtained for 10,000 replicates according to the "extended majority rule".

This type seems to be restricted to *Calystegia sepium* as alternative host plant and the vectors feeding on this plant.

The UPGMA tree (Fig. 3) shows the differentiation between the aster yellows isolate (AY) and the isolates of the stolbur group which corresponds with the results of the principal component analysis (Fig. 4). Within the stolbur group we obtained a differentiation of the isolate MOL. The phylogenetic analysis indicated a close relatedness between VK-Type II and III (Tab. 9, Figs 3 and 4). The VK-Type I was more similar to the isolate DEPL than to the VK-Types II and III. The bootstrap values obtained with the "extended majority rule" in Phylip indicated the most trustworthy phylogenetic tree but it showed also a high variance between pseudoreplicates. Tab. 10 shows the percentage of variance that could be explained by the first three components of the Principal Component Analysis.

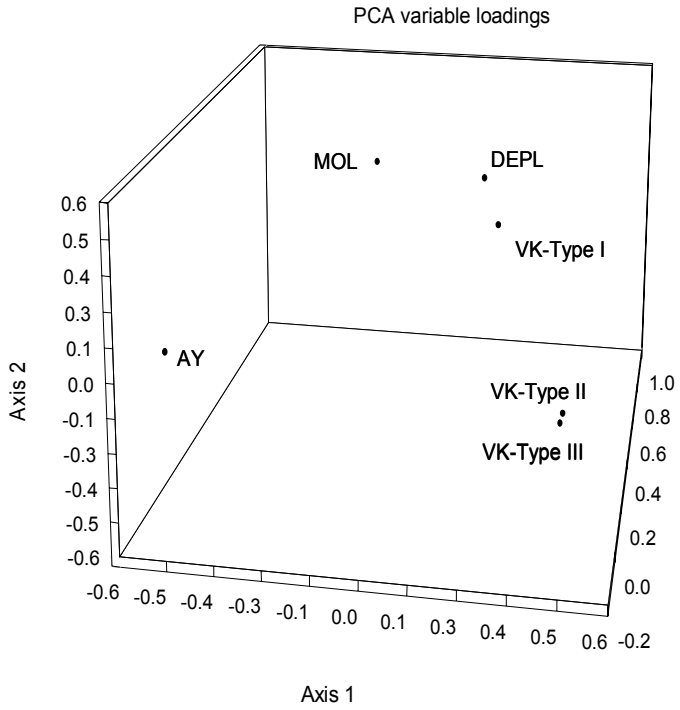

Fig. 4: Principal component analysis based on the genetic relatedness between the phytoplasma strains according to Lynch and Milligan (1994).

Table 9

Relatedness between pairs of phytoplasma strains according to Lynch and Milligan (1994) based on RFLP-data

	Type I	Type II	Type III	Moliere	DEPL
Type II	0.503				
Type III	0.515	0.863			
Moliere	0.428	0.331	0.342		
DEPL	0.631	0.534	0.397	0.460	
AV	0.021	-0.076	-0.064	0.592	0.053

Table 10

Eigen-values and cumulated percentage of three chosen components of the principal component analysis

	Axis 1	Axis 2	Axis 3
Eigen-values	0.52	0.12	0.03
Percentage	73.73	16.66	5.11
Cum. Percentage	73.73	90.39	95.5

Discussion

"Vergilbungskrankheit" is a widespread grapevine yellows in Germany. Closely related isolates have been distinguished previously by RFLP analysis of a non-ribosomal DNA-fragment (Reinert and Maixner 2000). They had been detected in grapevine as well as in the vectoring planthopper *H. obsoletus* and in herbaceous alternative host plants. One of these, *Convolvulus arvensis* (field bindweed), plays a key role in the epidemiology of VK in Germany. A new host plant of VK phytoplasma, hedge bindweed (*Calystegia sepium)*, was identified during this study. While field bindweed and stinging nettle (*Urtica dioica*) are long known as hosts of *H. obsoletus*, we proved the host plant status of *C. sepium* for the first time by collecting adult planthoppers from the leaves and larval instars from the roots of this plant.

The stolbur group has been shown to be quite homogenous. RFLP analyses of the 16S-rRNA gene revealed no differences between isolates from various hosts (Marcone *et al* 1996, 1997; Reinert and Maixner 2000). Differences have been found by RFLP- and sequence analyses of more variable non-ribosomal fragments (Marcone *et al*. 1999) and the comparison of genome sizes (Marcone *et al*. 1999). Reinert and Maixner (2000) distinguished two different groups of isolates based on RFLP-analysis of the non-ribosomal fragment Stol4 (Daire *et al*. 1997). A disadvantage of this procedure is the low sensitivity of the Stol4 primers that leads to a high proportion of false negative samples. This shortcoming could be overcome by the use of a second non-ribosomal fragment tufAY (Schneider *et al*. 1997 b) used in addition to Stol4.

We used various restriction enzymes to digest the two DNA fragments in order to obtain sufficient numbers of polymorphic fragments. RFLPs could be achieved by the digestion of the tufAY fragment with 4 and the Stol4 fragment with two enzymes. The data obtained by RFLP-analyses of both DNA-fragments were combined. Three different profiles (VK-type I, II, and III) could be obtained from grapevine samples and *H. obsoletus* respectively, while two of these profiles (II, III) were detected in *C. sepium.* Only one of the grapevine profiles was detected in *C. arvensis* (II) and another one in *U. dioica* (I). All periwinkle isolates of the

stolbur-group except for MOL and DEPL could be assigned to one of the profiles obtained from grapevine. Each of the latter two isolates showed an unique profile that was not detected in our field samples.

The cluster analysis proved the existence of three subgroups. VK types II and III clustered together. Besides isolates from grapevine, vectors, *C. sepium* and *C. arvensis*, also periwinkle isolates obtained from grapevine in Germany, Italy and Spain or from pepper in Serbia and tomato in France were assigned to this cluster. The second cluster consisted of VK type I together with periwinkle isolates obtained from grapevine in Italy and Germany and of the periwinkle isolate DEPL obtained from *Lavandula officinalis*. The periwinkle isolate MOL obtained from sour cherry in France was separated from these two groups.

The results of the cluster analysis are consistent with the findings of the principal component analysis. The first three components together explain more than 95 % of the total variance. Plotting of these components also led to three groups that were identical to the groups defined by cluster analysis. The significance of a classification of phytoplasmas on the basis of restriction profile polymorphisms without corresponding phenological or biological differences may be questionable. In the case of VK, however, the different types could be additionally characterised by their association to different herbaceous host plant species, while infected grapevines exhibited no evident differences with regard to the type and severity of symptoms. Beside grapevine, VK type I has only been detected in *U. dioica* and in *H. obsoletus* collected from this weed. On the other hand, type II which is the most widespread and abundant type in Germany is restricted to *C. arvensis* and *C. sepium*, while type III was detected in the latter weed only. The significance of type III is still unclear. So far we found it only in a restricted area in the lower Mosel valley while *C. sepium* populations in other areas were free of phytoplasmas or infected by VK type II. Since vector populations from nettle and bindweed show differences with regard to feeding preferences and the time required to complete their generation cycle (LANGER and MAIXNER, unpubl.) it could be hypothesised that different natural cycles of VK/BN exist in the field. While the system *C. arvensis* - *H. obsoletus* - *V. vinifera* is the predominant disease cycle in Germany (MAIXNER *et al*. 1995) and probably in France (SFORZA *et al*. 1998), the "nettle-system" (*U. dioica* - *H. obsoletus* - *V. vinifera*) might be more important in Italy, where *H. obsoletus* is reported to prefer stinging nettle (VIDANO *et al*. 1988; LESSIO *et al*. 2003). Although the epidemiology of VK/BN in Germany, France and Italy is well explained by these disease cycles, other models may exist in Mediterranean areas where *H. obsoletus* is present (Lebanon, Israel, Sicily, Spain), but no close association of the vector and the phytoplasma with the above mentioned host plants has been confirmed yet.

Previously VK type I was only known from the viticultural area of Palatinate. During our survey we found grapevines infected by this type in several viticultural areas and in unusual high incidence. It is not yet clear if this was due to a biased sampling or whether it indicated a spread of this type, probably caused by the hot and dry weather conditions in the previous years. VK type II is still the most frequent and most widespread in Germany, but the relative abundance of types I and III could increase due to environmental or cultural conditions.

The method described in this paper to characterise stolbur phytoplasmas could be useful for epidemiological studies but also for attempts to control the disease. If testing of symptomatic grapevines reveals the infection by stolbur phytoplasmas, the assignment of the pathogens to the VK types gives useful hints on the type of the predominant wild plant species that serve as reservoirs of the phytoplasma within or in the vicinity of affected vineyards.

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