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Invasion biology and host specificity of the grapevine yellows disease vector *Hyalesthes obsoletus* in Europe

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Abstract

Within the past 10 years, the yellows disease 'bois noir' (BN) has become one of the commercially most important diseases of grapevine [*Vitis vinifera* L. (Vitaceae)] in Europe. Infection pressure is caused by phytoplasmas of the stolbur 16SrXII-A group that are transmitted by a planthopper vector, *Hyalesthes obsoletus* Signoret (Homoptera: Auchenorrhyncha). Infestation happens as an accidental side-effect of the feeding behaviour of the vector, as vector and pathogen proliferation is dependent on other plants. In Germany, the increase of BN is correlated with the use of a new host plant by the vector, increase in abundance of the vector on the new host plant, and dissemination of host plant-specific pathogen strains. In this article, we investigate geographic and host-associated range expansion of the vector. We test whether host-plant utilization in Germany, hence the increase in BN, is related to genetic host races of the vector and, if so, whether these have evolved locally or have immigrated from southern populations that traditionally use the new host plant. The genetic population analysis demonstrates a recent expansion and circum-alpine invasion of *H. obsoletus* into German and northern French wine-growing regions, which coincides with the emergence of BN. No *H. obsoletus* mitochondrial DNA haplotype host-plant affiliation was found, implying that the ability to use alternative host plants is genetically intrinsic to *H. obsoletus*. However, subtle yet significant random amplified polymorphic DNA (RAPD) genetic differentiation was found among host plant populations. When combined, these results suggest that a geographic range expansion of *H. obsoletus* only partly explains the increase of BN, and that interactions with host plants also occur. Further possible beneficial factors to *H. obsoletus*, such as temperature increase and phytoplasma interactions, are discussed.

Introduction

Insect-transmitted phytoplasmas are responsible for diseases in hundreds of plant species (Lee et al., 2000). The trivial name 'phytoplasma' is used for wall-less, non-helical prokaryotes that colonize plant phloem and sap-sucking insect vectors (leafhoppers, planthoppers, and psyllids) and form a distinct, monophyletic clade within the class Mollicutes (Firrao et al., 2004). The epidemiology of phytoplasma-induced diseases is significantly influenced by the dispersal biology of their vectors and by the host spectrum of phytoplasma and vectors (Weintraub & Beanland, 2006; Firrao et al., 2007). Infection is thought to be determined largely by the number of vectors capable of transmitting the phytoplasma and the feeding mode of the vectors (monophagous vs. polyphagous) (Lee et al., 2000). Multiple hosts may enhance the opportunity for genetic exchange and local adaptations and give rise to various infectious pathways (Christensen et al., 2005). However, diversification of the vector's host plant range may lead to unsynchronized, partial vector–phytoplasma relationships where phytoplasmas are transmitted more to other plants than the normal reservoir. Such new relationships often entail increased virulence due to 'maladaptation' or competition between pathogen strains (Elliot et al., 2003).

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One type of partial relationship is experienced by so-called dead-end hosts. These are hosts on which vectors feed and subsequently infest, but from which the vector cannot acquire the pathogen (Weintraub & Beanland, 2006). The epidemiology of the disease on dead-end hosts is complex, due to an indirect infectious pathway shaped by extrinsic interactions between the vector, pathogen, and their common essential host plant(s). Grapevine [*Vitis vinifera* L. (Vitaceae)] is a dead-end host to phytoplasmas of the stolbur 16SrXII-A group (Lee et al., 1998) that cause the yellows disease 'bois noir' (BN). Phytoplasmas are accidentally transmitted to grapevine by the cixiid planthopper *Hyalesthes obsoletus* Signoret (Homoptera: Auchenorrhyncha) while it feeds on grapevine (Maixner et al., 1995). For *H. obsoletus*, grapevine is a suboptimal, erroneous food resource on which larval development does not take place. In a normal life cycle, phytoplasmas are acquired by *H. obsoletus* larvae via the roots of their common essential host-plant species and transferred back to these via the adult insect's saliva to the plant phloem. The study of BN is complicated by the difficulty of quantifying which plant species simultaneously act as host for *H. obsoletus* development and as pathogen reservoir. Field bindweed (*Convolvulus arvensis*) is generally considered both the primary host plant and reservoir in most countries (Suchov & Vovk, 1948; Blattný et al., 1954; Brčàk, 1955; Maixner et al., 1995; Sforza et al., 1999) but local patterns differ. For example, lavender (*Lavandula angustifolia*) is a common host in southern France (Leclant & Lacote, 1969; Sforza et al., 1999), but monk's pepper (*Vitex agnus-castus*) is a preferred plant in Israel (Sharon et al., 2005), while stinging nettle (*Urtica dioica*) is most likely favoured in Italy (e.g., Alma et al., 1987; Lessio et al., 2007).

During the last 10 years, BN has suddenly become one of the most important diseases of grapevine in Europe, with infection rates reaching 50–80% in some areas. Boudon-Padieu (1999) suggested that the spread and increase of BN is linked to geographical range expansion by *H. obsoletus*. This notion is appropriate for Germany, where the first record of *H. obsoletus* is from 1939 (Wagner, 1939). Symptoms compatible with BN were reported for the first time around this period, although the disease was attributed to nutrition deficiency (BN was not diagnosed until the 1960s). As late as 1986, *H. obsoletus* was considered extremely rare in Germany (Sergel, 1986), while BN [confused with Flavescence dorée (FD), another grapevine yellows that is not present in Germany] occurred locally at microclimatically favoured viticultural sites of the valleys of the Rhine and Mosel Rivers in the early 1960s and 1970s. Recent emergence of BN in areas peripheral to the central occurrence (Sabaté et al., 2003; Milkus et al., 2005; Sharon et al., 2005) further suggests geographic expansion.

The spread and increase of BN in Germany is, however, also linked to host-plant use. The traditional host plant of *H. obsoletus*, field bindweed, has within the last 10 years been replaced by stinging nettle as probably the most widespread host plant (Langer & Maixner, 2004). During this period, the abundance of *H. obsoletus* has increased extraordinarily. Langer & Maixner (2004) showed that vectors caught on stinging nettle and field bindweed harbour plant-specific stolbur strains. Furthermore, flight periods of *H. obsoletus* emerging from the alternative plants differ slightly (Maixner et al., 2006). Thus, the increase of BN since the 1990s in Germany coincides with the acquisition of a new host plant by the vector, an increase in abundance of *H. obsoletus* on the new host plant, and stolbur host plant-specific strains.

When combined, the above observations suggest two vector-related parameters associated to the recent spread of BN: (i) geographic and demographic expansion of *H. obsoletus* in Europe, and (ii) the existence of two host races, one of which specializes on bindweed and one on stinging nettle. In this article, we analyze these two possibilities by inferring historical dispersal of *H. obsoletus* using a phylogeographic approach, and we test putative host-race diversification of *H. obsoletus* among German and Slovenian host-plant populations using mitochondrial DNA (mtDNA) and random amplified polymorphic DNA (RAPD) markers. If host races occur in Germany, we consider whether they originated locally or are immigrants from southern populations that traditionally use the new host plant. Assuming that host races exist, we expect an in situ process when differentiation is independent of southern genealogical lineages but an immigration scenario if German host-plant populations are genealogically linked to southern populations from the same host plant.

Materials and methods

Collections – sampling scheme

Hyalesthes obsoletus was sampled from seven European countries (Germany, Spain, France, Italy, Slovenia, Austria, and Hungary) and Israel to test the immigration vs. in situ differentiation hypotheses. *Hyalesthes obsoletus* was sampled from the Convolvulaceae species *C. arvensis* (field bindweed) and *Calystegia sepium* (hedge bindweed) in Germany and Slovenia, and from *U. dioica* (stinging nettle) (Urticaceae) in all regions, except Israel where *H. obsoletus* was caught on olive (*Olea europaea*) and monk's pepper (*V. agnus-castus*). Mitochondrial genetic diversity was analyzed from all regions, while RAPD analyses were restricted to German and Slovenian populations and one Italian population, Rome. Sample localities are listed in Table 1.

Table 1 Sample localities and genetic polymorphisms in *Hyalesthes obsoletus*. n = sample size of mtDNA and random amplified polymorphic DNA (RAPD) data

			Coordinates		Number of mtDNA haplotypes													RAPD	
Locality	Country	Plant	North	East	n	aa	ab	af	bb	cd	db	ec	fc	ge	he	ib	ig	n	H_e
Bacharach	D	C	50°02'	07°46'	3	3												55[1]	0.33[2]
Bacharach	D	U	50°02'	07°46'	4	4												60[1]	0.28[2]
Bernkastel-1	D	C	49°54'	07°04'	4	4													
Bernkastel-2	D	U	49°54'	07°04'	4	4													
Kesten	D	Cal	49°54'	06°57'	2	2													
Kesten	D	U	49°54'	06°57'	1	1													
Kesten	D	C	49°54'	06°57'	1	1													
Lieser	D	C	49°55'	07°01'														18	0.24
Lieser	D	U	49°55'	07°01'														30	0.25
Graach	D	Cal	49°55'	07°03'														30	0.27
Lehmen	D	Cal	50°16'	07°27'	1	1													
Lehmen	D	Cal	50°16'	07°27'	1	1													
Ungstein	D	U	49°28'	08°11'	4	4												30	0.26
Randersacker	D	U	49°46'	10°00'														7	0.23
Thüngersheim	D	C	49°52'	09°50'	2	2													
Lahr	D	U	48°20'	07°27'	5	3			2										
Ortenau	D	U	48°31'	08°04'	2	1			1										
Turckheim	F	U	48°05'	07°16'	5	5												30	0.27
Longwy sur le Doubs	F	U	47°54'	05°21'	3				2	1									
Bretenieres	F	U	46°56'	05°32'	3				3										
Ambonil	F	U	44°47'	04°54'	1		1												
Montboucher sur Jabron	F	U	44°33'	04°48'	2		2												
near Pisa	I	?	43°42'	10°23'	3		1		1	1									
Verona	I	U	43°42'	10°59'	3		3												
Farra di Soligno	I	U	45°53'	12°06'	5		2	3											
Cordignano	I	?	45°56'	12°25'	5		3	1	1										
Rome	I	U	41°55'	12°25'	5		3									1	1	22	0.27
Aragon region	E	?	41°10'	01°00'	5		1		4										
Mark	SLO	U	45°56'	13°39'														9	0.25
Krsko	SLO	C	45°58'	15°28'	8	4	3					1						10	0.32
Jareninski	SLO	U	46°37'	15°41'	8	6	2											26	0.33
Kromberk	SLO	U	45°56'	13°39'	4		4											8	0.22
Strezetina	SLO	?	46°27'	16°09'														12	0.25
Deutschschützen	A	?	46°45'	15°58'	3	2	1												
Klöch	A	?	47°10'	16°27'	2	1	1												
Pécs	H	U	46°04'	18°13'	3							2	1						
Nagytótfalu	H	?	46°51'	18°20'	1							1							
Golan	IL	?	33°00'	35°50'	4									1	3				
Golan	IL	V	33°00'	35°50'	3									1	2				
Golan	IL	O	33°00'	35°50'	1										1				

Country: A, Austria; D, Germany; E, Spain; F, France; I, Italy; SLO, Slovenia; IL, Israel.

Plant: C, *Convolvulus arvensis*; Cal, *Calystegia sepium*; U, *Urtica dioica*; O, *Olea europaea*; V, *Vitex agnus-castus*; ?, no data.

[1]Total sample size for 2005 and 2006.

[2]Average gene diversity estimates for 2005 and 2006.

Specimens were collected in 2005 and 2006 by sweeping nets through stands of *C. arvensis* and *U. dioica*, respectively. At one location in Bacharach, Germany, *H. obsoletus* were sampled on both host plants in two consecutive years. All specimens from Germany and Alsace were sexed. Beside our own samples, we used insects collected by M. Breuer, Staatliches Weinbauinstitut (WBI), Freiburg; A. Schartl, Bayerische Landesanstalt für Weinbau und Gartenbau (LWG)

Veitshöchheim; and M. Stark-Urnau, Staatliche Lehr- und Versuchsanstalt für Wein- und Obstbau Weinsberg (LVWO) Weinsberg. *Hyalesthes obsoletus* from outside Germany were provided by E. Angelini, Istituto Sperimentale per la Viticoltura (ISV) Conegliano, Italy; A. Batlle, Institut de Recerca i Tecnologia Agroalimentaries (IRTA) Cabrils, Spain; E. Boudon-Padieu and Alberto Bressan, Institut National de la Recherche Agronomique (INRA) Dijon, France; P. Kuntzmann, Institut Francais de la Vigne et du Vin (ITV) Colmar, France; G. Pasquini, Istituto Sperimentale per la Patologia Vegetale (ISPV), Rome, Italy; H. Reisenzein and N. Zeisner Agentur für Gesundheit und Ernährungssicherheit, Institut für Pflanzengesundheit (AGES), Austria; G. Seljak, Agriculture and Forestry Institute, Nova Gorica, Slovenia; R. Sharon, Northern R&D, Kyriat Shemona, Israel; and D. Zsofia, Hungary.

mtDNA procedure

Total genomic DNA was extracted from whole animals using the High Pure Template Preparation kit (Roche Diagnostics, Mannheim, Germany). Individuals were analyzed for a double-stranded mtDNA template including four partial gene sequences (1180 base pairs). Ribosomal RNA (16S), tRNA(Leu) 217 bp, and reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit I (ND1) 193 bp were polymerase chain reaction (PCR) amplified using the primers LR-N-12945: 5'-CGA-CCT-CGA-TGT-TGA-ATT-AA-3' and N1-J-12261: 5'-TCG-TAA-GAA-ATT-ATT-TGA-GC-3' (Hedin, 1997). Cytochrome oxidase subunit I (CO I) (198 bp), tRNA(Leu) (67 bp) and cytochrome oxidase subunit II (CO II) (523 bp) were amplified using the primers S2792: 5'-ATA-CCT-CGA-CGT-TAT-TCA-GA-3' and A3661: 5'-CCA-CAA-ATT-TCT-GAA-CAT-TGA-CCA-3' (Brown et al., 1996). Gene amplification conditions were identical for the two primer pairs. Reactions were performed in an end volume of 25 µl, consisting of 1 µl forward and backward primer (10 pmol μl^{-1}), 1 µl DNA extraction, and 22 µl LiCrosolv® water (Merck, Darmstadt, Germany). Polymerase chain reactions were performed with PuReTaq Ready-to-Go beads (0.5 ml tubes; GE Healthcare Bio-Sciences, Uppsala, Sweden). The PCR was started by denaturing at 95 °C for 2 min, followed by 35 cycles of annealing at 48 °C for 1 min, extension at 72 °C for 1.5 min, and denaturation at 95 °C for 30 s. The PCR was terminated with a final extension at 72 °C for 2 min. Polymerase chain reaction products were sequenced using an ABI-377 (Perkin Elmer, Waltham, MA, USA) automatic sequencer.

Random amplified polymorphic DNA procedure

Random amplified polymorphic DNA analysis was performed on 347 individuals from six bindweed (five field bindweed and one hedge bindweed) and 10 stinging nettle populations using five arbitrary decamer primers: 04 (5'-AAGTCCGCT-3'), B4 (5'-GGACTGGAGT-3'), C7 (5'-GTCCCGACGA-3'), RAPD1 (5'-CTGCTGGGA-3'), and RAPD2 (5'-GAAACGGGTG-3') (Carl Roth GmbH and Co., Karlsruhe, Germany); these were chosen for amplifying with good band discrimination and consistently (repeatability) after initial primer trials with 10 primers (data not shown). Random amplified polymorphic DNA amplifications were performed in 25 µl volumes, containing Ready-To-Go™ PCR Beads, 3 µl primer (5 pmol μl^{-1}), 4 µl genomic DNA (all samples standardised 5 pmol μl^{-1}), and 18 µl deionised sterile water. The reaction mix was amplified with pre-treatment for 5 min at 95 °C, followed by 46 cycles of 1 min at 43 °C, 2 min at 72 °C, and 1 min at 95 °C. Amplification products were electrophoresed on 1.8% agarose gels at 120 V for 3 h, visualized with ethidium bromide, and photographed digitally under ultraviolet (UV) light. Fragment lengths were analyzed with the programme BioDocAnalyze (Whatman Biometra, Göttingen, Germany) by first laying reference lines estimated from a 100-bp DNA ladder (Invitrogen, Karlsruhe, Germany), and thereafter calibrating the lengths relative to reference *H. obsoletus* RAPD-PCR alleles. This procedure gave a conservative number of fragments, as fragments with minor length differences (which may be found within, as well as between, gels) were pooled. To further minimize PCR and gel artefacts, individuals from several populations were amplified and run simultaneously, and reference individuals on all gels were included.

Stolbur infestation and host-plant affiliation of *Hyalesthes obsoletus* in RAPD analysis

To make sure that host affiliation tests for *H. obsoletus* were not biased by amplifying RAPD bands from stolbur strains (which are plant specific), we compared RAPD bands amplified from infected and non-infected individuals of *H. obsoletus*. Infection was quantified by amplifying the stolbur-specific locus Stol-11 (F3/R2 primer) (see Clair et al., 2003) from genomic DNA extracted from individual *H. obsoletus*. Nettle and bindweed individuals from Bacharach in 2005 and 2006 (n = 115), Slovenia (n = 20), Lieser (n = 38), Graach (n = 20), and Rome (n = 20) were tested for stolbur infection.

Mitochondrial DNA statistical analysis

Haplotype mtDNA genealogies were calculated with the programme TCS 1.13 (Clement et al., 2000). Molecular diversity indices, pairwise differences (pd), and nucleotide diversity were calculated for each sample and for regional host-plant populations. Host plant-haplotype affiliations of *H. obsoletus* collected in Germany and Slovenia were quantified with the χ^2 test (JMP; SAS Institute, 1995).

Three alternative historical dispersal routes of *H. obsoletus* into Germany and North France (Alsace) from four putative regional sources (southern France, Spain, Italy, and Slovenia/Austria) were analyzed. Westwards immigration north of the Alps connected Germany to all regions via Slovenia; eastwards immigration south of the Alps connected Germany to Italian and Slovenian populations via southern France; multiple source immigration assumed linear routes from the nearest putative source (island model). The three scenarios were tested under the assumption of isolation by distance (IBD) between regional samples. All regions were subjectively based on the geographic location of sampling, and for the Alsace population due to its combined geographic and genealogical position. Hungarian and Israeli populations were phylogenetic out-groups and not included in IBD analysis (see Results). Geographic distances for IBD analysis were calculated between the geographic centres of the regional samples. Genetic distances were based on pairwise F_{ST} estimates [analysis of molecular variance (AMOVA) – pairwise distance matrix] calculated with Arlequin version. 3.11 (Excoffier et al., 2005). Isolation by distance was tested by regressing genetic and geographic distances (JMP; SAS Institute, 1995) and with the Mantel permutation test (1000 permutations, subroutine Isolde in Genepop version 3.4) (Raymond & Rousset, 1995). Both IBD tests were performed on observed distances and transformed distances [genetic distance: F_{ST} and $F_{ST}/(1 - F_{ST})$; geographic distance: km; and ln (km)]. Population expansion was evaluated by testing the observed mismatch distribution for significant deviation from a sudden population expansion model calculated with Arlequin version 3.11 (Excoffier et al., 2005).

Random amplified polymorphic DNA statistical analysis

Random amplified polymorphic DNA genetic diversity was analyzed with the programme TFPGA (Miller, 1997). Allele frequencies were calculated assuming Hardy–Weinberg proportions as the square root of the frequency of the absent allele. We employed four procedures for testing host-plant fidelity based on the presence/absence of RAPD bands. First, levels of gene diversity (Nei's unbiased heterozygosity; Nei, 1978) and percentage polymorphic loci (95% level of polymorphism) were compared among host plant-affiliated populations. Mean population estimates were compared with an unpaired t-test. In Bacharach, between-year and between-host gene diversity was tested locus-by-locus using paired t-tests. Frequency data were square root arcsin transformed before being tested. t-Tests were performed with JMP (SAS Institute, 1995).

Second, interindividual relationships and host-plant fidelity were examined in multidimensional space by principal coordinates analysis (PCO) using the programme PCO (Anderson, 2003). The PCO was based on a squared Euclidean distance matrix, and coordinate scores were projected in two dimensions. PCO analysis was performed at three levels: (i) for all individuals (n = 347), (ii) separately on northern individuals ([n = 260; 103 bindweed and 157 stinging nettle), and (iii) *H. obsoletus* from Bacharach, Germany, where insects from both host plants were sampled in two consecutive years (n = 115; 55 bindweed and 60 stinging nettle).

Third, hierarchical population structure was calculated with inbreeding indices (F-statistics) with TFPGA. Variance was estimated relative to host-plant affiliation and geographic origin, where F_{ST} is the variance among all populations at a given geographic level of investigation, F_{HT} is the variance between host-plant populations, and F_{CT} = is the variance between countries of origin. The host-plant analysis was done for all, northern (Germany and Alsace), and Bacharach populations, separately. Country level analysis was performed between German (including the French Alsace population) and Slovenian/Italian populations. Hierarchical analyses of F_{ST} (and PCO) were not performed separately for Slovenian populations, because only a single bindweed population was sampled in Slovenia. This circumstance means that any random perturbation of this bindweed population (e.g., bottleneck) would produce a biased variance estimate in favour of host-plant fidelity. An estimate of the Slovenian bindweed population affinity was gained from the fourth host-plant test, population relationships using maximum likelihood analysis based on RAPD allele frequencies (subroutine CONTML in PHYLIP version 3.6; Felsenstein, 1993). This method assumes that branch lengths are determined by drift alone.

Results

Mitochondrial DNA genetic diversity and phylogeography

The 1180 characters assayed from 111 *H. obsoletus* individuals revealed 12 compound haplotypes (Table 1) with 26 segregating sites [GenBank accession numbers EU155640-48 (COI-tRNA(Leu)-CO II) and EU155649-55 (16S-tRNA(Leu)-ND1)]. Haplotype diversity was lowest in Germany ($n_{individuals} = 34$, $n_{haplotypes} = 2$, mean number of pd = 0.331, and nucleotide diversity $\pi = 0.00030$) and highest in Italy ($n_{ind} = 21$, $n_{hapl} = 6$, pd = 0.971, and $\pi = 0.00083$), followed by (in descending order): Slovenia, $n_{ind} = 20$, $n_{hapl} = 3$, pd = 0.760, and $\pi = 0.00064$; Spain and France (considered as one region), $n_{ind} = 14$, $n_{hapl} = 3$, pd = 0.582, and $\pi = 0.00047$; Hungary, $n_{ind} = 5$, $n_{hapl} = 2$, pd = 0.500, and $\pi = 0.00042$; and Israel, $n_{ind} = 8$, $n_{hapl} = 2$, pd = 0.428, and $\pi = 0.00038$.

The haplotype genealogy was fully resolved, showing high concordance with geography (Figure 1). A major

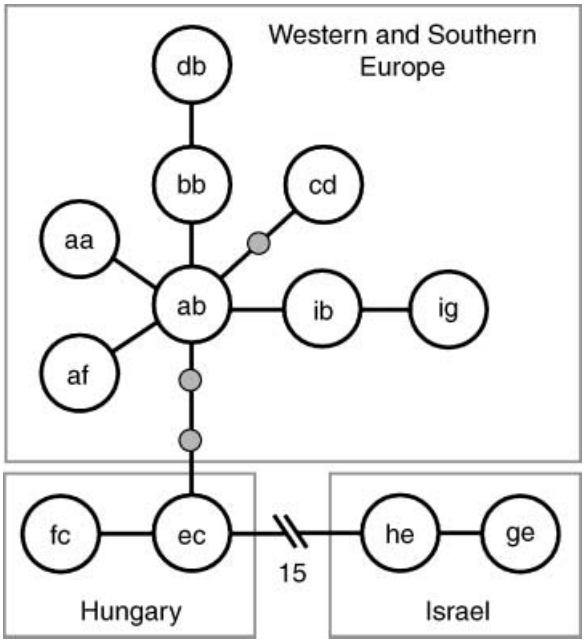

Figure 1 Haplotype network of *Hyalesthes obsoletus* haplotypes (1180 bp), showing three biogeographic clades: (i) a western and southern European clade including individuals from Slovenia, (ii) a Hungarian, and (iii) an Israeli clade. One 'Hungarian' haplotype was found in Slovenia.

phylogenetic partition (15 mutations) was observed between Israel and European specimens. Two sublineages were observed within Europe, an 'eastern' sublineage in Hungary and a 'western' sublineage in western and northern Europe. The basal western European haplotype (ab) was rooted to the Hungarian haplotypes. The haplotype ab was observed throughout southern Europe. The western sublineage showed a star-like gene tree with two frequency-based phylogeographic borders. First, the derived haplotypes aa and bb were found on alternative sides of the classical Slovenian/Italian limestone biogeographic divide, and, second, these derived haplotypes meet at a contact zone in southern Germany, strongly suggesting circum-alpine dispersal into Germany/North France from a historical source in the northern Balkans or northern Italy (Figure 2).

An original westwards immigration into Germany and northern France (i.e., north of the Alps via Slovenia) was supported by significant IBD between regions (Mantel test: $P = 0.01$; regression analysis: $r^2 = 0.61$, $P<0.01$). No IBD was found for eastwards immigration (i.e., south of the Alps via Italy and southern France) (Mantel test: $P = 0.26$; regression analysis: $r^2 = 0.05$, $P = 0.84$), or for direct immigration (independent sources and across the Alps) (Mantel test: $P = 0.26$, $r^2 = 0.03$, $P = 0.74$). The mismatch distribution of the western European clade did not deviate significantly from a sudden expansion model, sum of squared deviation (SSD) = 0.00278, $P = 0.20$.

Host-plant affiliation – mtDNA

No host plant-haplotype dependency was observed in Germany ($\chi^2 = 0.26$, $P = 0.61$) or Slovenia ($\chi^2 = 0.03$, $P = 0.86$) (Table 2). German populations were near monomorphic for the aa haplotype, which was found on both host plants. The bb haplotype in Germany was found

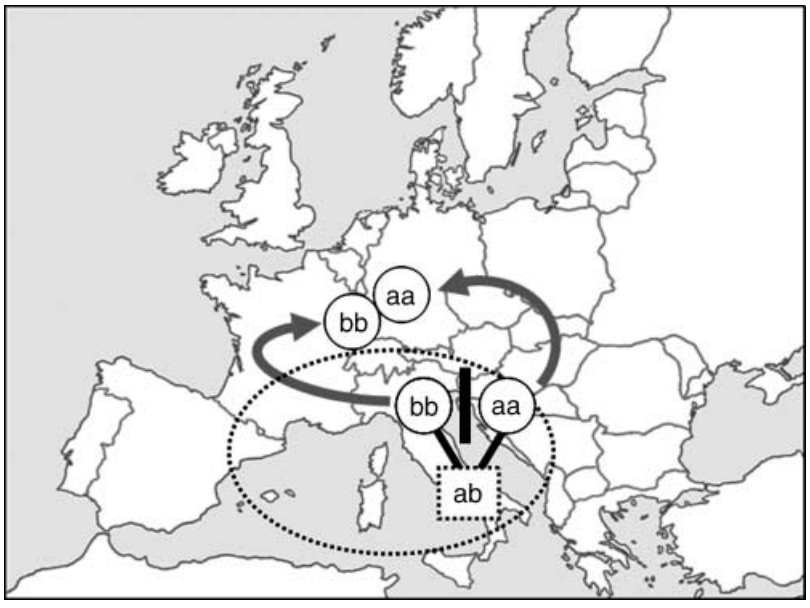

Figure 2 Circum-alpine dispersal of *Hyalesthes obsoletus* observed from geographic distributions and genealogy of the three common western clade haplotypes aa, ab, and bb. The ancestral haplotype ab was found throughout southern Europe (dotted circle). Haplotypes aa and bb were found on either side of the Italian-Slovenian limestone divide (bold line), and meet at a secondary contact zone in southern Germany.

Table 2 Association between *Hyalesthes obsoletus* mtDNA haplotypes and host-plant affiliation (*Convolvulus arvensis* and *Urtica dioica*) in Germany and Slovenia

Germany	Haplotypes		Slovenia	Haplotypes		
Plant	aa	bb	Plant	aa	ab	ec
U. dioica	21	3	*U. dioica*	6	6	0
C. arvensis	10	0	*C. arvensis*	4	3	1
Total	31	3	Total	10	9	1

in the Southwest in three stinging nettle individuals only. As bb is probably new to Southwest Germany (see above), and as no bindweed animals were analyzed from this area, we could not determine plant affinity for bb haplotypes. In Slovenia, the basal western ab haplotype as well as its derived aa haplotype were found on both plants.

Host-plant affiliation – RAPD analysis

The five RAPD primers amplified a total of 59 scorable fragments: 56 fragments were polymorphic, with 10–15 polymorphic fragments generated per primer. All RAPD fragments were found in infested and non-infested *H. obsoletus* alike in equal proportions. Bacharach and Lieser were the only two samples with more than three infected individuals (Bacharach: 15 infected bindweed, 26 infected nettles; Lieser: seven infected bindweed, eight infected nettles). Thus, no stolbur-specific fragments were found and all RAPD fragments were included in analysis.

No fragments were diagnostic for plant-affiliated populations. Mean gene diversity per population was influenced by sample size in stinging nettle ($r^2 = 0.32$, $P<0.05$), but not in bindweed ($r^2 = 0.03$, $P = 0.80$) populations. No correlation was found in stinging nettle if locations with less than 11 individuals were omitted. Therefore, we based between-host variability tests on populations with more than 11 individuals. Among all populations, the percentage of polymorphic loci per population was significantly higher in bindweed (0.75; $n = 5$) than stinging nettle (0.67; $n = 8$) populations ($t = 2.26$, d.f. = 11, and $P = 0.045$). The trend was similar among German populations (bindweed 0.75; $n = 4$; stinging nettle 0.66; $n = 5$), but due to unequal variances the differences could not be tested.

Bindweed populations had higher gene diversity at all geographical levels, but not significantly so (mean estimates all populations: bindweed $H_e = 0.30$, stinging nettle $H_e = 0.26$; Germany: bindweed $H_e = 0.29$, stinging nettle $H_e = 0.26$). At Bacharach, gene diversity estimates of bindweed populations (2005: $H_e = 0.31$; 2006: $H_e = 0.35$) were higher than stinging nettle populations (2005: $H_e = 0.27$; 2006: $H_e = 0.28$) and significantly so in 2006 (locus per locus paired t-test, $P<0.01$). However, the 2006 result is ambiguous as a host-plant indicator, because gene diversity in bindweed differed significantly between years before Bonferonni correction ($P = 0.023$). Stinging nettle populations did not differ between years ($P = 0.40$).

Principal coordinate analysis from a squared Euclidean distance matrix among RAPD phenotypes was performed for three data sets: all individuals, northern individuals (Germany, Alsace), and Bacharach individuals. In each analysis, the first three coordinates never accounted for more than 20% of the total variance. The first PCO axis never explained more than 10% of the total variance. PCO analysis did not discriminate host-plant affiliation in the total data set. In contrast, host-plant affiliation was observed for the northern and Bacharach data sets, albeit that axis scores were very low. The Lieser bindweed population displayed an aberrant banding pattern by lacking bands at the 04 primer. Analysis with Lieser bindweed animals separated bindweed animals from stinging nettle animals on positive PCO I and PCO II scores (9.85 and 5.23%, respectively). An analysis omitting Lieser bindweed also divided bindweed and stinging nettle populations, with populations separated due to negative PCO II scores (5.61%) in bindweed animals. Host-plant separation in Bacharach (PCO I = 8.12%, PCO II = 6.00%) (Figure 3) was influenced neither by sex nor year.

RAPD genetic variance among host-plant populations at the total and northern level of investigation was low but significant, all populations: $F_{HT} = 0.025$ (0.005–0.049, 95% interval), $F_{ST} = 0.174$ (0.147–0.204); northern populations (Germany, Alsace): $F_{HT} = 0.037$ (0.008–0.069), $F_{ST} = 0.169$ (0.139–0.203), while host-plant variance increased at

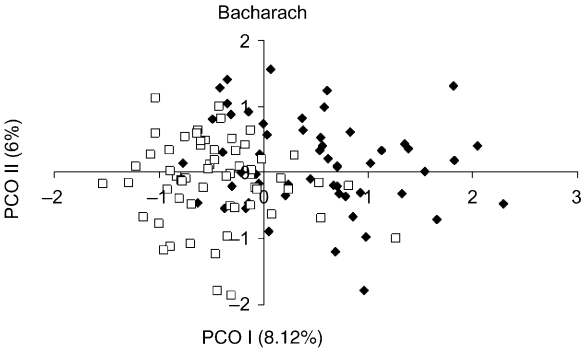

Figure 3 Plot of the first (PCO I) vs. second (PCO II) principal coordinate for 115 multilocus random amplified polymorphic DNA (RAPD) phenotypes of *Hyalesthes obsoletus* sampled at Bacharach, Germany, in 2005 and 2006 from field bindweed (solid diamonds) and stinging nettle (open squares).

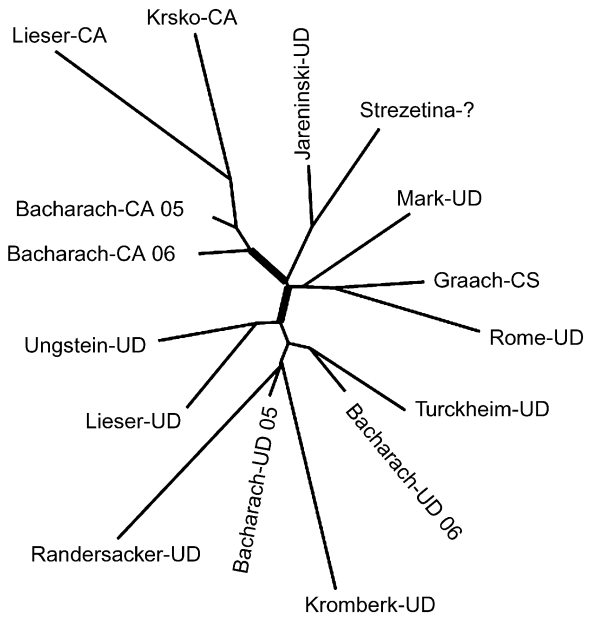

Figure 4 Maximum likelihood phylogenetic tree of *Hyalesthes obsoletus* bindweed and stinging nettle populations based on random amplified polymorphic DNA (RAPD) data. Interior bold lines indicate significant divisions between field bindweed populations, German stinging nettle populations, and Slovenian/Italian stinging nettle populations. CA, field bindweed; CS, hedge bindweed; and UD, stinging nettle.

Bacharach relative to the higher geographic levels, $F_{HT} = 0.064$ (0.029–0.100), and variance between all samples decreased, $F_{ST} = 0.107$ (0.071–0.147). Differentiation between northern (Germany, Alsace) and Slovenian/Italian populations was not significantly greater than 0, $F_{CT} = 0.022$ (–0.001–0.049).

Maximum likelihood phylogenetic analysis based on RAPD allele frequencies found three significant splits representing general, though not perfect, host-plant and geographic divisions: (i) field bindweed populations, (ii) Slovenian/Italian stinging nettle populations, and (iii) German stinging nettle populations (Figure 4).

Discussion

The incidence of BN in Europe has increased dramatically during the last decade. The spread involves a complex interaction between dispersal and proliferation of the vector, and several (probably unknown) reservoir plants of the phytoplasma. If BN-stolbur is transmitted solely by *H. obsoletus*, the question arises whether *H. obsoletus*, and with BN, is expanding its range from a source population(s) or whether it was always present in low numbers but is now increasing from local stocks due to local intrinsic factors?

Historical dispersal

Here, we show that southern and western European populations of *H. obsoletus* have a common origin and that they expanded from a single source population in a circum-alpine manner to northern central Europe. Germany was originally colonised from the eastern haplo-lineage aa. The mtDNA data strongly suggest the Italian-Slovenian area to be the geographic origin of the clade. Italian populations harbour the highest diversity, whereas the geographic proximity of Slovenia to Hungary, where insects have the ancestral haplotype (ec) of the western mtDNA clade, may place Slovenia as the origin of the western clade. The division between *H. obsoletus* in Israel and Europe and the Hungarian haplotypes indicate that *H. obsoletus* had a Levantine origin, colonizing Europe through southeast Europe. However, these eastern lineages were not responsible for the current expansion in western Europe. Diversification within the western clade entailed evolution of the derived haplotypes aa and bb on either side of the traditional limestone phylogeographic barrier between Italy and Slovenia. The circum-alpine range expansion of *H. obsoletus* is now complete: the haplotypes aa and bb meet at a secondary contact zone in southern Germany/northern France. Lack of mtDNA diversity in Germany and no variability at 13 enzyme loci (B Lux, unpubl.) corroborates recent colonization in this region. These findings strongly suggest that the emergence of BN in Germany since the 1960s is caused by very recent immigration of *H. obsoletus*.

Host-plant specialization

Although *H. obsoletus* is a recent faunal element in Germany, the immigration model alone cannot explain all of the contemporary spread of the disease. *Hyalesthes obsoletus* has been present in the Mediterranean (Hoch & Remane, 1985) before severe outbreaks happened here. It seems that the interaction between phytoplasma, host plants, and *H. obsoletus* has changed as well. The question is, therefore, if *H. obsoletus* has only recently acquired a phytoplasma strain that is spreading with the vector or whether local environmental and biotic parameters have changed, allowing the spread of disease. Parameters that might facilitate the spread of *H. obsoletus* are (i) the evolution of host races with a distinct biology, (ii) environmental changes, for example, higher ambient temperatures allowing host-range expansion (with or without host specialization), and (iii) fitness benefits for infected vectors. In this regard, range expansion of *H. obsoletus* (and BN) is much more complex than that of the grapevine yellows FD vector *Scaphoidus titanus*, which is restricted to grapevine. The spread of *S. titanus* and FD in Europe can be explained by a diffusion process due to trade of grapevine canes and grafts (Bertin et al., 2007).

In Germany, where BN is well documented, the recent spread of BN correlates with the use of a new host plant by the vector and the colonization of additional habitats where this new host plant is available. Although our data are suggestive of host specialization, genetically very similar host-plant populations make their genetic coherency unclear; it is also unclear whether specialization itself is related to the increase of BN. On the one hand, the derived aa and basal ab mtDNA haplotypes were both not affected by host-plant differentiation, implying an intrinsic ability of maternal lineages to colonize both plants. On the other hand, RAPD data indicated subtle yet significant host-plant diversification on a large scale (maximum likelihood tree) and local geographic scale (PCO and F-statistics). Furthermore, in Bacharach, where both host plants were sampled in two consecutive years, host plant-affiliated populations differed genetically, in size (wing length) (K Michel, unpubl.), and in flight phenologies (Maixner et al., 2006). Although data from one population alone are only suggestive of host races (phenotypic differences might be environmentally and not genetically determined), the combination of genetic and ecological diversification points in this direction. Shallow diversification in RAPD and mtDNA rules out old host races of *H. obsoletus*, but the subtle diversification leads us to hypothesize on-going host-plant specialization.

Host-plant expansion of *H. obsoletus* in Germany is probably related to an increase in ambient temperatures that has made it possible for *H. obsoletus* to shift to a hitherto suboptimal host (larvae survive on roots) either due to an increased larval growth rate or because of a longer feeding period as a result of the extended growing season (Boudon-Padieu & Maixner, 2007). Abundance related to temperature was documented for *H. obsoletus* in the Golan Heights in Israel (Orenstein et al., 2003). During the last 20 years, the mean annual temperature has increased by 1.4 °C in the Mosel valley. A temperature-dependent host-plant expansion may rest on the two alternatives outlined previously: (i) in situ temperature increase, or (ii) an influx of southern nettle-adapted *H. obsoletus*. The former is possible, because *H. obsoletus* seems to harbour intrinsic genetic variation for nettle colonization; the latter is possible, because southern nettle populations are migrating north and may have entered the bindweed cycle recently. Maternally inherited mtDNA may not reveal the latter. As often observed in univoltine insects, males emerge earlier than females. Because bindweed insects emerge first, nettle males encounter, on average, bindweed females first. If the two mate, mtDNA from bindweed insects will dominate, and will not reveal influx of (nuclear) genetic variation permitting adaptation to nettle. Because mtDNA in our study did not resolve host specificity, and as adequate southern French sample sizes for RAPD analysis were lacking, we presently cannot discriminate between the two alternatives. Fortunately, our study has identified two areas that present promising conditions for clarifying the process of diversification and plant adaptation, namely, the division area between Italy and Slovenia and the contact zone in Germany/France.

Vector–stolbur interactions

Regarding *H. obsoletus*, the in situ temperature scenario has one shortcoming explaining the epidemiology of BN in Germany: it does not explain why different stolbur strains are found only on stinging nettle (strain I) and field bindweed (strain II), respectively. If rising temperatures have allowed *H. obsoletus* to expand its host range, allowing *H. obsoletus* to only recently colonize a new host, host fidelity should not be perfect and stolbur strains should mix. The questions are whether (i) strains have diverged during acquisition of the novel host by *H. obsoletus*, (ii) strain specificity is a sign of phytoplasma host races, or (iii) both strains are found on the original host (field bindweed) but fitness of strain I is markedly higher on the novel host (stinging nettle). The first assumption seems unlikely as stolbur type I is more related to lavender stolbur than to stolbur type II (Langer & Maixner, 2004), and preliminary stolbur sequence data show four strain-specific mutations (J Johannesen, unpubl.). The number of mutations seems too high to have arisen during a recent host shift. The mutational differences imply host races of stolbur (question ii). In this case, transmission between nettles may involve an original, alternative vector and be indicative of a host shift of *H. obsoletus*. Question (iii) may indicate but does not necessarily require host races of *H. obsoletus*; it needs only an environmental change permitting *H. obsoletus* to colonize a new host – the stolbur strains survive according to where they are accidentally transmitted but exhibit differential fitness, and thus dominate, on either of the two host plants. Presence of both strains in one plant may not be detected due to a PCR bias in favour of the dominant strain. Questions (ii) and (iii) both imply acquisition of new stolbur strains by *H. obsoletus*, which might affect the fitness of *H. obsoletus*.

Elliot et al. (2003) showed that virulence to the reservoir or the vector may depend on habitat heterogeneity and mobility. The general assumption that parasites should become benign towards the vector is predicted when it is the more mobile species, free parasites rarely disperse, and parasite strains do not compete (Elliot et al., 2003). These assumptions seem likely for *H. obsoletus* and stolbur. Stolbur are known only to disperse via the vector. No information is available about competition between

stolbur strains, but it might be low or non-existent as the two strains (I and II) have never been found together in the alternative host plant (reservoir) (M Maixner, pers. obs.). However, no quantitative host races of *H. obsoletus* would restrict competition between stolbur strains. Fitness benefits to infected vectors may include increased longevity and fecundity (Beanland et al., 2000) and survival (Ebbert & Nault, 2001; Belliure et al., 2005). The latter authors hypothesized that the pathogen (a virus) helps the vector overcome plant defences against their arthropod vectors. However, reduced fitness due to phytoplasma infection has been reported from phytoplasma vectors as well. Bressan et al. (2005) reported significant reduction of fecundity and longevity in *Scaphoideus titanus* infected by FD phytoplasma. Garcia-Salazer et al. (1991) observed that fitness of the vector *Paraphlepsius irroratus* infected with X-disease phytoplasma was temperature dependent and the lifespan of this vector was significantly reduced in the natural temperature range. Interestingly, bindweed, the traditional reservoir plant in Germany, shows clear stolbur-related symptoms, whereas stinging nettle does not. Does this indicate that stinging nettle has a specific stolbur strain that has been set free by a host shift of *H. obsoletus*? At present, this remains speculation but may serve as a future research objective for understanding the epidemiology of bois noir.

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