Host race formation in *Hyalesthes obsoletus* (Signoret 1865)

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Zusammenfassung

Die Winden-Glasflügelzikade *Hyalesthes obsoletus* (Cixiidae, Glasflügelzikaden) nutzte in Deutschland ursprünglich die Ackerwinde *Convolvulus arvensis* als Wirtspflanze, allerdings nahm in den letzten zwei Dekaden die Abundanz auf der Großen Brennnessel *Urtica dioica* stark zu, zusammen mit der Inzidenz der Schwarzholzkrankheit Bois noir auf Weinreben. Bois noir wird durch ein Phytoplasma verursacht, das durch *H. obsoletus* von *C. arvensis* und *U. dioica* auf Weinreben übertragen wird. Es stellte sich daher die Frage, ob *H. obsoletus* Wirtsrassen entwickelt hat, die möglicherweise die Bois noir-Epidemiologie beeinflussen. In der vorliegenden Studie wurden folgende Fragestellungen bearbeitet:

(1) Gibt es in Deutschland und Europa genetisch unterscheidbare Wirtsrassen von *H. obsoletus* auf den beiden Wirtspflanzen *C. arvensis* und *U. dioica*? Es wurden sieben Mikrosatellitenmarker entwickelt und etabliert, um *H. obsoletus* Populationen aus Deutschland und Europa genetisch zu analysieren. Es zeigte sich eine deutliche Differenzierung zwischen Populationen von beiden Wirtspflanzen in Deutschland, jedoch nicht in den historischen Ursprungsgebieten der deutschen Populationen, in der Schweiz, Italien oder Slovenien.

(2) Wo sind die deutschen Wirtsrassen von *H. obsoletus* entstanden? Eine Einwanderung von südlichen, bereits an *U. dioica* angepassten Individuen stand einer lokalen Wirtsrassenevolution gegenüber. Die engere genetische Verwandtschaft der deutschen Population auf *U. dioica* zu denen auf *C. arvensis*, im Vergleich zu den übrigen Populationen auf *U. dioica*, impliziert einen lokalen Prozess im nördlichen Verbreitungsgebiet. Eine Immigration südlicher Tiere scheint nicht zur Diversifizierung beigetragen zu haben, führte aber möglicherweise einen *U. dioica*-spezifischen Phytoplasma-Stamm ein. Durch Wirtsrassenevolution entwickelten sich spezifische, vektorbasierte epidemiologische Kreisläufe der Schwarzholzkrankheit Bois noir.

(3) Welche Präferenzen zeigen die beiden Wirtsrassen von *H. obsoletus* für die Wirtspflanzen *C. arvensis* und *U. dioica* und unterscheiden sich diese? Die Präferenz von *H. obsoletus* aus beiden deutschen Wirtsrassen in Bezug auf den Geruch der Wirtspflanzen wurde in einem Y-Olfaktometer untersucht, zusätzlich wurden beide Pflanzen direkt zur Wahl gestellt. Bei beiden Untersuchungen zeigte die Population von *C. arvensis* eine signifikante Präferenz für ihre native Wirtspflanze. Die Population von *U. dioica* wies dagegen keine Präferenz für den Geruch einer Wirtspflanze auf, bevorzugte im direkten Test jedoch signifikant ihre native Wirtspflanze. Dies weist darauf hin, dass die Anpassung an den „neuen" Wirt noch nicht vollständig ist.

Outline

In this dissertation, I investigate the host race formation in *Hyalesthes obsoletus*, using genetic and ecological approaches. In a review, I first give a short general introduction into the evolution of host races and evaluate the characteristics of *H. obsoletus* that make it susceptible for host race formation. I summarize the conducted analyses and put them in a broader perspective. The analyses are presented in three research articles (see below), which are presented as chapters 1 to 3 and referred to by their respective numbers.

Chapter 1 deals with the development of microsatellite markers for the further genetic analyses of *H. obsoletus*' population structure. Chapter 2 presents a thorough genetic analysis of *H. obsoletus* populations from Europe. Chapter 3 presents two ecological approaches to analyze the host preference of German *H. obsoletus*.

(Chapter 1) Imo M, Lüneburg J, Hankeln T, Seitz A, Johannesen J (2011) Highly polymorphic di- and trinucleotide microsatellite markers for the grapevine yellows disease vector *Hyalesthes obsoletus* (Cixiidae). European Journal of Entomology, 108, 161–163.

(Chapter 2) Imo M, Maixner M, Johannesen J (2013) Sympatric diversification vs. immigration: Deciphering host-plant specialization in a polyphagous insect, the stolbur phytoplasma vector *Hyalesthes obsoletus* (Cixiidae). Molecular Ecology (in press)

(Chapter 3) Imo M, Maixner M, Johannesen J, Gross J (2013) Bindweed or nettle: Do the genetic host races of *Hyalesthes obsoletus* differ in their preference? (in preparation for publication)

Review

Host race formation in *Hyalesthes obsoletus*

Abstract

Host races are often considered an intermediate stage on the continuum leading from phenotypic plasticity to full species. The evolution of host races is particularly prevalent in species that have an intimate interaction with their hosts, like phytophagous insects. In this review, I explore the process of host race formation in the polyphagous planthopper *Hyalesthes obsoletus* (Signoret 1865; Homoptera: Auchenorrhyncha). Host race formation in this species is of major economic interest, since the population structure of *H. obsoletus* is tightly connected with the epidemiology of the *H. obsoletus*-transmitted grapevine yellows disease bois noir.

Studies using genetic and ecological approaches provide strong evidence for the local formation of host races of *H. obsoletus* in Germany. Populations of *H. obsoletus* associated with the two major hosts in Midwestern Germany exhibited a significant genetic differentiation. However, no genetic differentiation has been found in the southernmost German population as well as in the remaining distribution range of *H. obsoletus*, including its native range in Italy and Slovenia. Results of the ecological studies showed a strong preference for the native host of the populations associated with the original host in Germany. Populations associated with the novel host, however, exhibited ambiguous results. When presented with whole plants, they significantly preferred their native host, but when presented with volatiles only, they chose randomly. Putting these results into broader perspectives, I discuss the evidence for a locally restricted evolution of host races and the potential relevance of host race formation for the epidemiology of transmitted diseases.

Introduction

The most successful species over the long run are the ones most adaptable to change. A heterogeneous environment and changes in abiotic factors promote heterogeneity in organisms, and therefore increase biodiversity. Variations in response range along a

continuum from the evolution of phenotypic plasticity to the point of speciation, whereas intermediates along this continuum are for example genetic polymorphism between interbreeding individuals or the development of semi-isolated biotypes and races. Forces such as genetic drift and gene flow determine whether a species is stable or moves along this continuum (Dres & Mallet 2002; Peccoud *et al.* 2009).

The environment of many organisms is largely comprised of another organism, their so-called hosts. Some are restricted to one host throughout their lifetime, while others switch between hosts of the same or even different species. Polyphagous species are able to utilize different host species, but preference hierarchies often differ between populations and even within populations and may also change with the availability of preferred species or other factors like climate conditions (Johnson *et al.* 1996; Rice 1984; Singer *et al.* 1989). When gene flow is restricted between populations utilizing different hosts, host races may arise. Many definitions for host races have been proposed, for example by Bush (1969), Diehl & Bush (1984), Jaenike (1990), Dres & Mallet (2002), and Coyne (2004); here I use the term host race in the sense of genetically distinct, sympatric populations that use different host species, but exhibit appreciable gene flow, as defined by Dres and Mallet (2002).

The polyphagous cixiid planthopper *Hyalesthes obsoletus* Signoret (Homoptera: Auchenorrhyncha) is native to southern Central Europe, the Mediterranean, South-Russia, Kazakhstan and Asia Minor. *Hyalesthes obsoletus* is xerotherm and lives in fallow ground and ruderal habitats, disturbed areas in calcareous grassland, usually on poriferous soil (Biedermann & Niedringhaus 2004). At its northern range limit, in Germany and northern France (Alsace) (Hoch and Remane 1985), it is usually found in vineyards at the steep slopes of river valleys. *Hyalesthes obsoletus* prefers herbaceous plants and its main hosts in Germany are *Convolvulus arvensis* (field bindweed) and *Urtica dioica* (stinging nettle), but preferences for certain host species vary regionally (e.g. Alma *et al.* 1987; Leclant & Lacote 1969; Lessio *et al.* 2007; Maixner *et al.* 1995; Sforza *et al.* 1999; Sharon *et al.* 2005).

Given the intimate associations with its host plants, host race formation may be an important force generating diversity in this species. In this review, I evaluate the characteristics of *H. obsoletus* that may make it susceptible to the evolution of host races. First, I briefly review theoretical concepts linked to the evolution of host races and why I might expect host races to arise in *H. obsoletus*. I then review current evidence for host race formation of this species via the studies in chapter 1, 2 and 3 that examine population genetics and behavioral ecology.

Evolution of host races

Host races represent an intermediate step along the continuum from genetic polymorphism to sister species. Polymorphisms, host races, and species are distinguishable in the pattern and often the number of differences, which depend on the amount of gene flow between the groups. This continuous range of sympatric biotypes provides evidence for the continuous route towards sympatric speciation via natural selection (Dres & Mallet 2002).

Host races are defined as genetically distinct, sympatric populations of parasites that utilize different hosts and between which appreciable gene flow, more than about 1 % per generation, exist (Dres & Mallet 2002). There is no postmating hybrid incompatibility among races, but they show habitat-dependent fitness tradeoffs (Bush 1994).

One key factor in the evolution of host races and initiation of sympatric speciation via habitat shift is the evolution of genetically based differences regarding host preference and fitness (Bush 1994). The original population utilizes only one host, but mutations lead to a shift in host preference in single individuals. When new alleles for host preference arise, a host shift may take place. Feeding and mating in the preferred habitat can then lead to genetically distinct habitat or host races and eventually to full sister species. In this case, isolating barriers are initially induced by genetic variation, not by distance, which proves sympatric speciation (Coyne & Orr 2004).

In general, host races are expected to be quite common among phytophagous insects, however, proven cases are rather rare. Speciation events may occur too quickly to detect the intermediate stage of host races frequently in nature. Many polyphagous insect species may actually consist of several sympatric, monophagous biotypes (Dres & Mallet 2002; Magalhaes *et al.* 2007). Host races can also be unstable stages in generalist species, which rarely evolve into distinct species. Generalists could as well be a recompilation of to date unidentified host races. Specialists, on the other hand, could be the result of host race formation (Magalhaes *et al.* 2007).

Why expect host-race formation in *Hyalesthes obsoletus*

There are several characteristics that may promote the evolution of host races, many of which are present in *H. obsoletus*.

Low dispersal ability and philopatry

A lack of active dispersal favors population divergence (Magalhaes *et al.* 2007). *Hyalesthes obsoletus* is a species with rather low dispersal abilities; active dispersal seems to be limited to neighboring plants within a distance of about ten meters (J. Johannesen & M. Maixner, unpublished data). Especially the females seem to move around very little, as usually only males are caught with yellow sticky traps (M. Maixner, unpublished data). It has also been observed that there is a high incidence of the grapevine yellows disease bois noir at the border of vineyards, close to strips of nettle harboring *H. obsoletus*, which is a known vector of the disease. But the vectors are unable of great dispersion into the vineyards (Bressan *et al.* 2007). Thus, *H. obsoletus* seems to be quite philopatric, which may facilitate specialization. Long-distance dispersal could occur passively via wind. In this case, new hosts will most likely be colonized by few individuals, which may diverge rapidly. Depending in the suitability and cost of adapting to the newly acquired host, this divergence may subsequently result in host race formation (Magalhaes *et al.* 2007).

High intimacy with the host environment

Herbivorous insects are considered prime candidates for sympatric speciation via host race formation because of their close intimacy and often strong specialization on their host plants, which usually comprise their living, feeding, and mating site (Via 2001). *Hyalesthes obsoletus* also exhibits a close relationship with its host plant. Eggs are laid in July and August close to the host plant. Within four weeks the eggs develop into nymphs, which feed on the roots of herbaceous plants and stay in the soil until the next spring. Emergence of adult *H. obsoletus* starts – depending on climate conditions – between the beginning of June and end of July. Adults feed on phloem sap of their host plants and also mate on the hosts (Langer & Maixner 2003). It is therefore most likely that individuals with the same preference for a certain host meet and mate on this host and eggs are laid in the soil next to this host.

Differences in phenology

Hyalesthes obsoletus associated with *U. dioica* emerge about two to four weeks after those associated with *C. arvensis* (Lux *et al.* 2006). Since the activity period of individuals is rather short, individuals that emerged from the same host are more likely to interbreed than individuals emerging from different hosts. The different phenology and host-associated mating should lead to assortative mating and subsequent genetic differentiation due to genetic drift.

Different phytoplasma strains

Phytoplasmas are obligate symbionts of plants and insects (Hogenhout *et al.* 2008). They are wall-less, insect-transmitted bacteria that cause more than 700 diseases in hundreds of plant species (Weintraub & Beanland 2006). One of them is the grapevine yellows disease bois noir, caused by the stolbur Sr16XII phytoplasma. The main host plants of *H. obsoletus*, *C. arvensis* and *U. dioica*, also serve as hosts for the stolbur phytoplasma. *Hyalesthes obsoletus* is the only confirmed vector of the stolbur phytoplasma to grapevine (Maixner *et al.* 1995; Sforza *et al.* 1998). Nymphs and adults of *H. obsoletus* acquire the phytoplasma by feeding on the phloem of infected plants. The phytoplasmas move through and replicate in the competent vector's body and circulate in the hemolymph (Weintraub & Beanland 2006). When *H. obsoletus* adults suck on noninfected plants, they transmit the phytoplasma. Since *H. obsoletus* feeds from time to time on grapevine, even though grapevine is not a potential host, it transmits the pathogen. Grapevine is a dead-end host for the phytoplasma; stolbur cannot be acquired by *H. obsoletus* from grapevine. Once infected with the stolbur phytoplasma, grapevine develops the typical bois noir-symptoms (rolling and discoloring of the leaves, blackening of the shoots, shriveling of the berries, sour and bitter taste of the berries). Increasing incidence of bois noir in German vineyards within the last two decades causes immense economic loss. Langer and Maixner (2004) found that *C. arvensis* and *U. dioica* harbor different stolbur strains – tuf type b and a, respectively. *Hyalesthes obsoletus* associated with either plant accordingly harbors the respective stolbur type (Figure 1). A double infection with both strains has so far not been shown. The existence of two genetically distinct stolbur strains associated with the two host plants suggests that the vector transmitting this pathogen exhibits two host races as well.

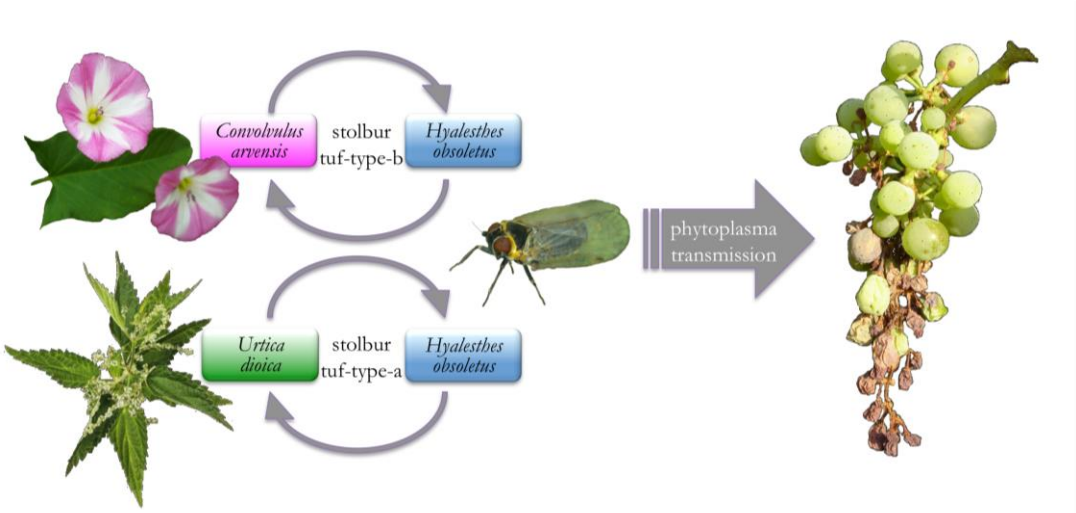

Figure 1 Epidemiological cycles of bois noir. *Hyalesthes obsoletus* associated with *C. arvensis* acquire stolbur tuf-type-b phytoplasma as nymphs while feeding on *C. arvensis* roots and transmit it as adults to uninfected *C. arvensis* when feeding on the phloem. Accordingly, *H. obsoletus* associated with *U. dioica* acquire stolbur tuf-type-a from infected *U. dioica* and transmit it to uninfected ones. Both stolbur strains can be transmitted to grapevine during accidental feeding.

Evidence for host-race formation in *Hyalesthes obsoletus*

I have tested the existence of host races in *H. obsoletus* in two ways: (1) Genetic studies using molecular markers (chapter 1 and 2), and (2) ecological studies using two-choice experiments with field populations (chapter 3). I compared my own analyses and experiments with studies conducted by colleagues.

Genetic studies using molecular markers

Molecular markers can reveal host-associated divergence in sympatric as well as allopatric populations. To show that divergence has resulted from some type of host adaptation and to rule out isolation by geographic distance, it should be shown that the genetic distance among populations from the same host type, even between allopatric populations, is lower than that of sympatric populations from different host types (Magalhaes *et al.* 2007).

To detect incipient phenomena such as host race formation, genetic markers need to be chosen that exhibit a sufficiently high polymorphism and are selectively neutral. AFLPs and microsatellites exhibit a much higher polymorphism than allozyme markers or

mitochondrial DNA (Magalhaes *et al.* 2007), but only microsatellites are considered reasonably neutral (Jarne & Lagoda 1996).

So far, the population structure of *H. obsoletus* has been analyzed by mitochondrial DNA and random amplified polymorphic DNA (RAPD). Johannesen *et al.* (2008) proposed a recent expansion and circum-alpine invasion of *H. obsoletus* into German and northern French wine-growing regions. Among host populations, there was no mitochondrial DNA haplotype host-plant affiliation to be found, but a subtle, yet significant RAPD genetic differentiation.

Highly polymorphic genetic markers that are selectively neutral were required for detecting recent changes in the population structure. Therefore, we established species-specific microsatellite markers (chapter 1). Seven markers, which showed reliable results and a sufficiently high polymorphism, were chosen for further analyzes of *H. obsoletus* population structure.

Using these newly developed microsatellite markers, I attempted to answer the following questions: a) Did *H. obsoletus* evolve host races? b) Are host races in *H. obsoletus* a universal or a locally restricted phenomenon? c) What factors might have facilitated the host race evolution of *H. obsoletus*?

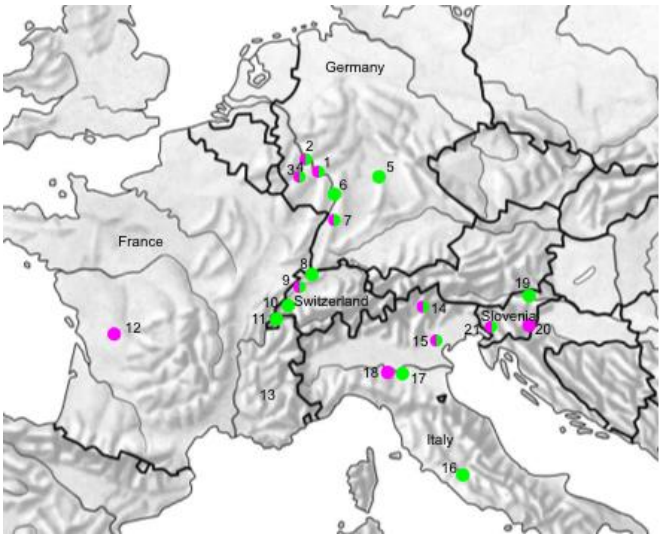

Figure 2 Sample locations in Germany, France, Switzerland, Italy and Slovenia. Shown are locations with *C. arvensis*-associated populations (pink), *U. dioica*-associated populations (green) and syntopic populations (split circles). For location numbers see Table 1. Map modified from www.swisseduc.ch.

Microsatellite analyses were conducted with *H. obsoletus* population samples collected from *C. arvensis* and *U. dioica* in Germany, France, Switzerland, Italy, and Slovenia (Figure 2). Additionally, *H. obsoletus* collected from further host plants were included in the analyses: from *Lavandula angustifolia* from France, from *Solanum tuberosum* from Romania and Russia, and from *Vitex agnus-castus* from Israel (Table 1). Hierarchical analysis of the data revealed a strong and highly significant ($F_{HT} = 0.054$, $p < 0.001$) genetic differentiation

between populations associated with *C. arvensis* and *U. dioica* in mid-western Germany. However, there was no evidence for host races in the southernmost German location or in the remaining regions, including the native regions of *H. obsoletus*, Italy and Slovenia. Furthermore, genetic diversity was reduced in populations in mid-west Germany associated with *U. dioica* relative to those associated with *C. arvensis*. The latter displayed a similar genetic diversity as the populations in the contact zone and in the native region. German *U. dioica*-associated populations were genetically more similar to German *C. arvensis*-associated populations than to populations from southern Europe, which are adapted to *U. dioica*. Taken together, there is strong evidence for a primary local diversification of *H. obsoletus* in mid-west Germany. German *U. dioica*-associated populations are monophyletic and diversification is affecting all seven loci equally, which implies that the host shift was a single event (chapter 2).

Table 1 Overview of all samples used in this thesis. *C.a.* = *Convolvulus arvensis*, *U.d.* = *Urtica dioica*, *L.a.* = *Lavandula angustifolia*, *S.t.* = *Solanum tuberosum*, *V.a.-c.* = *Vitex agnus-castus*

Country	Site	Sample year	*N* on *C.a.*	*N* on *U.d.*	*N* on *L.a.*	*N* on *S.t.*	*N* on *V.a.-c.*	Location on map (Fig. 2)	Included in chapter
Germany	Bacharach	2005	22	27	-	-	-	1	2
Germany	Bacharach	2006	29	29	-	-	-	1	2
Germany	Bacharach	2007	20	20	-	-	-	1	1 & 2
Germany	Bacharach	2008	20	20	-	-	-	1	2
Germany	Bacharach	2009	20	20	-	-	-	1	2
Germany	Bacharach	2011	34	-	-	-	-	1	3
Germany	Boppard	2007	7	13	-	-	-	2	2
Germany	Boppard	2011	98	-	-	-	-	2	3
Germany	Boppard	2012	400	-	-	-	-	2	3
Germany	Lieser	2005	18	-	-	-	-	3	2
Germany	Lieser	2006	-	30	-	-	-	3	2
Germany	Bernkastel-Kues	2006	-	24	-	-	-	2	2
Germany	Bernkastel-Kues	2008	22	-	-	-	-	4	2
Germany	Bernkastel-Kues	2011	82	300	-	-	-	4	3
Germany	Kesten	2011	-	120	-	-	-	4	3
Germany	Kesten	2012	-	400	-	-	-	4	3
Germany	Randersacker	2006	-	5	-	-	-	5	2
Germany	Ungstein	2006		30	-	-	-	6	2
Germany	Neuweier	2009	22	10	-	-	-	7	2
Switzerland	Arlesheim	2008	-	9	-	-	-	8	2
Switzerland	Le Landeron	2010	23	24	-	-	-	9	2

Country	Site	Sample year	*N* on *C.a.*	*N* on *U.d.*	*N* on *L.a.*	*N* on *S.t.*	*N* on *V.a.-c.*	Location on map (Fig. 2)	Included in chapter
Switzerland	Morges	2009	-	10	-	-	-	10	2
Switzerland	Russin	2008	-	10	-	-	-	11	2
France	Charentes	2009	22	-	-	-	-	12	2
France	Mévouillon	2009	-	-	18	-	-	13	2
Italy	Eisacktal. Feldthurns	2007	15	10	-	-	-	14	2
Italy	Treviso	2006	6	6	-	-	-	15	2
Italy	Rom	2006	-	18	-	-	-	16	1 & 2
Italy	Modena	2009	-	16	-	-	-	17	2
Italy	Reggio Emilia	2009	8	-	-	-	-	18	2
Slovenia	Jareninski	2004	-	6	-	-	-	19	2
Slovenia	Krško	2004	7	-	-	-	-	20	2
Slovenia	Nova Gorica	2006	25	19	-	-	-	21	2
Romania	Fundulea	2008	-	-	-	14	-	not shown	2
Romania	Radovanu	2009	-	-	-	14	-	not shown	2
Russia	Mayak	2009	-	-	-	20	-	not shown	2
Israel	Golan	2006	-	-	-	-	20	not shown	1 & 2

Ecological studies using field populations

A better performance on and a preference for the original host indicate host-linked specialization (Kawecki 2004). Ideally, it should be controlled for maternal effects and previous experience of organisms. Unfortunately, this was not possible in the ecological studies using *H. obsoletus*, which cannot be successfully reared over several generations in the lab. Despite these limitations, in combination with the genetic analyses, the conducted ecological studies still provide important insights into the formation of host races in *H. obsoletus*.

In two two-choice experiments (chapter 3), I tested *H. obsoletus* from mid-western Germany, the region where a strong genetic differentiation between the host races has been shown (Figure 2, locations 2 and 4; Table 1). Using a Y-shaped olfactometer, single individuals were given the choice between the odor of the two main host plants, *C. arvensis* and *U. dioica*, or between either plant and pure air. Specimens collected from *C. arvensis* showed a strong preference for their native host. However, *U. dioica*-associated specimens did not exhibit a significant preference for either host. Female specimen even showed a non-significant tendency to prefer the ancestral host *C. arvensis*.

Additionally, I tested the preference of 800 *H. obsoletus* in groups of 20 individuals of the same sex in cages with the two hosts *C. arvensis* and *U. dioica*. Again, specimens collected from *C. arvensis* showed a strong preference for their native host. But contrary to the olfactometer setup, specimens collected from *U. dioica* also significantly preferred their native host (chapter 3).

Did the stolbur phytoplasma influence host-race evolution in *Hyalesthes obsoletus*?

Phytoplasmas have been shown to render plants more suitable for phloem-feeders or even convert non-hosts into hosts or better hosts for phloem-feeding insects (reviewed in Hogenhout 2008). *Hyalesthes obsoletus* specimens used for the ecological tests (chapter 3) were tested for a stolbur infection. No significant influence of the stolbur phytoplasma on survival or host choice could be detected, however, the low infection rate of about 20 % renders the sample size very small. The olfactometer setup indicated a slight preference of infected *H. obsoletus* collected from *U. dioica* for their native host. A higher sample size is necessary to reveal whether this tendency actually is a preference or mere coincidence. The influence of host plant infections on *H. obsoletus* could not be tested due to technical constraints. Especially for *U. dioica*, it's very difficult to artificially infect plants and to verify an infection, since *U. dioica* does not show any symptoms and infection could not be proven genetically (Albert 2011). An improvement of the methods and a follow-up of the ecological analyses contrasting healthy with infected plants and healthy with infected vectors would provide valuable insights into the possible influence of the stolbur phytoplasma on host-race evolution in *H. obsoletus*.

Conclusion and perspectives

Genetic analyses strongly implied that a local host race formation took place in German *H. obsoletus* populations and that *U. dioica* is the novel host. If the host shift took place rather recently, the ancestral population should exhibit a strong preference for their original host, but the diverged population on the novel host might not (yet) have developed a preference for this host. In this case, genetic differentiation would merely be maintained by assortative mating caused by different phenologies and low dispersal, not by active choice. These expectancies have been confirmed in the two-choice experiments. However, a longevity experiment (Albert 2011) did not completely match these results. Although, as expected, each population survived longer on its native host, *U. dioica* seemed to be the species that provides higher fitness for *H. obsoletus*. On the other hand, the lower growth

rate on *U. dioica* was assumed to render *U. dioica* a less ideal host. How do these results go together? A lower growth rate does not necessarily imply reduced fitness. It may even induce increased fitness if it is compensated by a longer feeding period, made possible by an increased growing season of the host. A slower growth rate may require less energy and therefore spare more energy for reproduction and longevity. *Urtica dioica* may actually not be - as has been assumed - a nutritionally less ideal host for *H. obsoletus*. This novel host just may not have been suitable due to suboptimal climate conditions in the northern distribution range of *H. obsoletus*. When mean annual temperatures rose and growing season extended, *U. dioica* became a suitable host. Those individuals that started to adapt to this host, maybe facilitated by an influx of a genetically pre-adapted individuals from southern Europe, may now experience a fitness benefit. The assumption that *U. dioica* is actually the better host for *H. obsoletus* is supported by the primary use of *U. dioica* in southern Europe, even when both hosts are available.

The ecological experiments revealed that *C. arvensis*-associated *H. obsoletus* always prefer their native plant, whether they are presented with only an olfactory clue or the whole plant. This indicates that olfactory clues play a major role in host recognition. Contrary, *U. dioica*-associated *H. obsoletus* only preferred *U. dioica* when the whole plant was presented. Their decision may have depended on visual clues or actual probing of the phloem sap. When presented with only olfactory clues, they seemed to choose randomly. However, their choice may not have been solely random. Another explanation is that part of the population still prefers *C. arvensis* odor, while the other part already prefers the odor of the new host *U. dioica*. If other factors like visual clues and probing of phloem sap are of greater importance for host discrimination, it is reasonable that these traits are under stronger selection. If this was the case, we expect that adaptation to *U. dioica* is not yet completed. The preference for *U. dioica* odor should grow stronger in the future because individuals with a preference for the "wrong" odor may waste energy by probing the less ideal host before realizing their "mistake".

Chapter 1

Highly polymorphic di- and trinucleotide microsatellite markers for the grapevine yellows disease vector *Hyalesthes obsoletus* (Cixiidae)

Abstract

Seven polymorphic microsatellite loci were developed for the planthopper *Hyalesthes obsoletus*, vector of stolbur 16SrXII-A phytoplasma. The loci have di- or trinucleotide repeat motifs and are highly variable with 10 to 22 alleles per locus. Observed heterozygosity ranged from 0.278 to 0.950 for the 78 individuals genotyped. One locus is sex-linked. No linkage between loci was found. All loci amplified consistently among phylogeographic as well as host-plant related samples and proved highly informative for population genetic studies.

Introduction

Epidemiological cycles of vector-transmitted diseases are greatly influenced by the dispersal ability and the host specificity of the vector (Weintraub & Beanland 2006). The polyphagous planthopper *Hyalesthes obsoletus* is the main vector of stolbur 16SrXII-A phytoplasma, a pathogen of many Solanaceae crops and responsible for the economically important grapevine yellows disease bois noir in Europe. The epidemiology of bois noir is primarily determined by the abundance of the vector on field bindweed (*Convolvulus arvensis*) and stinging nettle (*Urtica dioica*) (Maixner *et al.* 1995). Phenological differences in the life cycle of the vector on these two plants (Maixner *et al.* 2009) and the presence of plant-specific stolbur strains (Langer & Maixner 2004) indicate host-races of vector and host-strains of the pathogen, and hence two epidemiological disease cycles. However, the ability to test for genetic host races of the vector and determine its dispersal ability has been hampered by the extremely little genetic polymorphism in the markers so far analyzed (mtDNA, RAPD-DNA, allozymes). This lack of polymorphism is most likely due to founder effects resulting from recent range expansion into large parts of the current

European distribution (Johannesen *et al.* 2008). In the present paper, the development of microsatellite genetic markers that facilitate the study of the evolution of host races in *H. obsoletus* and the epidemiology of *H. obsoletus*-transmitted diseases are reported.

Materials and Methods

Genomic DNA was purified (DNeasy Kit, Qiagen, Hilden, Germany) and sheared by nebulization. The size fraction between 1 and 2.5 kb was electro-eluted from a 1 % agarose gel, end-repaired using Klenow enzyme and T4 DNA polymerase, blunt-end ligated into dephosphorylated, SmaI-digested pUC 18 plasmid vector and transformed into electro-competent E. coli DH10B cells (Amid *et al.* 2001). After blue-white selection, about 1000 white colonies were analyzed for the presence of microsatellite-containing inserts by colony filter hybridization. Synthetic oligonucleotides representing a mixture of the various di- and trinucleotide motifs (30 mers) were radio-labelled using T4 polynuclotide kinase and gamma-P32-ATP (Hartmann Analytic, Braunschweig, Germany). Hybridization was performed at 40°C in 6 x SSC/1 % sodium-dodecylsulfate overnight, followed by washes at 50 °C in 2 x SSC and 1 x SSC (1 x SSC contains 0.015 M trisodium citrate and 0.15 M sodium chloride). Autoradiography was performed using Kodak X-Omat films (Sigma-Aldrich, St. Louis, USA).

Plasmid DNA from positive clones was isolated in a 96-well format (Qiagen) and sequenced by the Sanger method using DyeTerminator chemistry (Applied Biosystems, Weiterstadt, Germany). Sequencing reactions were separated on an ABI3730 sequencer by a commercial service (StarSeq, Mainz, Germany). Bioinformatic identification of microsatellite stretches was performed with the program SciRoKo (Kofler *et al.* 2007).

Eighteen loci with repeat motifs were isolated (GenBank Accession no. HM046814 - HM046831). For fluorescent labelling, the cost-efficient one-tube single-reaction nested PCR method described by Schuelke (2000) was used first. An 18-bp M13 primer was added to the 5′ end of each forward primer and a fluorescent-labelled M13 primer was added to the PCR. PCR amplification was performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Munich, Germany) following PCR conditions described in Schuelke (2000). Cycling conditions were: 5 min at 94 °C, 30 cycles of 30 sec at 94 °C, 45 sec at 55/59/60/64 °C, 45 sec at 72 °C, followed by 8 cycles of 30 sec at 94 °C, 45 sec at 53 °C, 45 sec at 72 °C, with a final extension of 15 min at 72 °C. Samples were scored on an ABI3130 sequencer using 11.7 µl HiDi formamide, 0.3 µL ROX 500 standard (Applied

Biosystems) and 1 µl of the PCR product. Loci were genotyped using GeneMapper 4.0 software (Applied Biosystems).

Loci that produced consistent results were amplified in two QIAGEN Multiplex PCR reactions with four and three fluorescent labelled primers, respectively (mix 1: B82, F56, F84 and H120, annealing temperature 60 °C; mix 2: E96, G85 and C147, annealing temperature 62.5 °C). For the multiplex PCR, a PCR volume of 10 µl (8.5 µl mastermix and 1.5 µl DNA of c. 50 ng DNA per reaction) was used. The mastermix contained a final concentration of 1x QIAGEN Multiplex PCR Master Mix, which provides 3 mM MgCl2, and 0.2 µM of each primer. Cycling conditions were: 30 sec at 95 °C, 30 cycles of 30 sec at 94 °C, 90 sec at 60/62.5 °C, 90 sec at 72 °C, followed by a final extension of 10 min at 72 °C.

Genetic variability and amplification consistency was tested in two German, one Italian and one Israel population. German populations (Bacharach) were syntopic and collected on *Urtica dioica* (N = 20) and *Convolvulus arvensis* (N = 20), respectively. Italian specimens (N = 18) were collected near Rome on *Urtica dioica*. Israel specimens (N = 20) were collected at several sites in the Central Golan Heights on *Vitex agnus-castus*. Genetic diversity indices, Hardy–Weinberg probabilities and population differentiation were calculated with GENEPOP version 4.0.10 (Raymond & Rousset 1995). Genetic differentiation among populations, F_{ST}, was calculated using the method of Weir & Cockerham (1984). Null alleles were tested using MICRO-CHECKER version 2.2.3 (van Oosterhout *et al.* 2004) applying a Bonferroni correction for multiple comparisons (Rice 1989).

Results

Seven polymorphic microsatellite loci produced consistent results. These loci amplified consistently in all 78 specimens. 10-22 alleles per locus (mean 15.86) were recorded. The expected heterozygosity per locus ranged from 0.533 to 0.941 (Table 2). The loci B82, F56, F84 and H120 obeyed Hardy-Weinberg proportions in all populations. Locus C147 was sex-linked; males were always homozygous whereas females showed Hardy-Weinberg proportions. Locus E96 showed deviations from Hardy-Weinberg proportions in two populations and locus G85 showed significant but similar levels of homozygote excess in all populations.

Table 2 Characteristics and summary statistics for microsatellite loci for *H. obsoletus* based on 78 individuals from four populations sampled in Germany (D-U [*U. dioica*], N = 20, and D-C [*C. arvensis*], N = 20), Italy (I), N = 18, and Israel (IL), N = 20.

Locus						D-U		D-C		I		IL	
GenBank Accession no.	Repeat motif	Primer sequence (5'-3')	T_a[1] (°C)	No. of alleles D-U/D-C/I/IL/total	Allele size range (bp)	H_O[2]	H_E	H_O	H_E	H_O	H_E	H_O	H_E
Hob B82 *HM046815*	$(CA)_{10}$	F: TGTAAGCACAAGAGACCTATCG R: CTCTTCCCTTCGTCAACG	59	7/11/10/14/17	131-171	0.650	0.754	0.650	0.826	0.778	0.843	0.800	0.905
Hob C147[3] *HM046818*	$(CA)_{21}$	F: GGTGTTTTTCCTCTACTGTCTGAG R: GGACATGGCTACGTTCAACA	60	5/11/8/13/18	141-205	0.750	0.569	0.900	0.901	0.556	0.744	0.278*	0.941
Hob E96 *HM06828*	$(GA)_{15}$ (interrupted by four T)	F: CGGCGTAAATTTGGAGAGAA R: ATCCCTCTTCCCCTTCTTCA	64	4/10/8/16/22	160-218	0.550	0.671	0.647	0.775	0.529*	0.818	0.526*	0.895
Hob F56 *HM046823*	$(CCA)_{12}$	F: AAGGCGCACGTTTCTACTGT R: TCGAAAATCGCGTTATCAGTC	60	8/9/5/13/14	164-215	0.700	0.681	0.650	0.812	0.500	0.692	0.950	0.873
Hob F84 *HM046824*	$(CA)_{13}$	F: CCACCTTTTCCCCTTATGAA R: GAGACTCCAGTTGCCACACA	60	5/8/3/10/10	237-260	0.700	0.628	0.950	0.792	0.556	0.584	0.600	0.794
Hob G85 *HM046828*	$(CA)_{10}$	F: AGCAAACACCTGCCTCTGAA R: CCAAAATTAGCGAACCGAAC	64	6/8/6/15/18	236-272	0.526*	0.771	0.400*	0.699	0.353*	0.533	0.650*	0.927
Hob H120 *HM046830*	$(CA)_3TA(CA)_7$	F: AACTCTCATGCGGACCAGAC R: AAGGGGATGGGTAGAACGAC	60	5/7/8/11/12	239-267	0.750	0-638	0.850	0.803	0.944	0.814	0.684	0.886

[1]T_a = annealing temperature. [2]GENEPOP v4.0.10 (Raymond & Rousset 1995) was used to calculate H_O (observed heterozygosity), H_E (expected heterozygosity). [3]Sex linked locus: The heterozygosity estimates for the German population were based only on females, whereas the sex of Italian and Israel populations were unknown. * denotes significant deviations from Hardy-Weinberg proportion.

The estimate of genetic differentiation among all populations was high for polymorphic microsatellite loci, F_{ST} = 0.12. Pairwise F_{ST} values are shown in Table 3. All F_{ST} values were highly significant ($p < 0.0001$).

Table 3 Pairwise F_{ST} values based on 78 individuals of *H. obsoletus* from four populations sampled in Germany (D-U [*U. dioica*], N = 20, and D-C [*C. arvensis*], N = 20), Italy (I), N = 18, and Israel (IL), N = 20. Overall F_{ST} = 0.12. All F_{ST} values were highly significant ($p < 0.0001$).

	D-U	D-C	I
D-C	0.12		
I	0.15	0.11	
IL	0.15	0.07	0.13

Bayesian clustering analysis performed with STRUCTURE 2.1 (Pritchard *et al.* 2000) for individuals sampled on different host plants at the syntopic German site clustered all individuals, except one, according to the host plant on which they were collected (proportion of membership to bindweed = 0.915, nettle = 0.936).

Discussion

The microsatellite loci reported here amplify consistently in *H. obsoletus* from diverse phylogeographic areas and host-plants. An overall departure from Hardy-Weinberg equilibrium was found for one locus, G85. Since there were no amplification failures at G85, and because the heterozygote deficit was constant across the four divergent populations, this indicates that a null-allele cannot alone explain the deficit (putative null allele frequencies = 0.11 - 0.16). No linkage was found between any locus pair, therefore all loci can be considered as independent. The Israel population was the only one with an overall deviation from Hardy-Weinberg proportions. This might be caused by the Wahlund effect as no amplification failures were observed. The Wahlund effect is a reduction in expected heterozygosity of a population due to structuring into several subpopulations. Israel specimens where collected at several sites in the Central Golan Heights and might therefore not represent a single population.

The loci were able to detect population differentiation within and between geographic regions, as is indicated by high F_{ST} values ranging from 0.07 to 0.15. The Israel population is phylogenetically divergent from German and Italian populations, which belong to two

related but separate post-glacial expanding lineages (Johannesen *et al.* 2008). In Germany, populations from different host plants showed significant genetic differentiation. The evidence provided here for geographic as well as host-plant related genetic variance show that the seven loci are highly informative for gaining insights into both host-race evolution in *H. obsoletus* and epidemiological cycles of *H. obsoletus*-transmitted diseases.

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Chapter 2

Sympatric diversification vs. immigration: Deciphering host-plant specialization in a polyphagous insect, the stolbur phytoplasma vector *Hyalesthes obsoletus* (Cixiidae)

Abstract

The epidemiology of vector transmitted plant diseases is highly influenced by dispersal and the host-plant range of the vector. Widening the vector's host range may increase transmission potential whereas specialization may induce specific disease cycles. The process leading to a vector's host shift and its epidemiological outcome is therefore embedded in the frameworks of sympatric evolution versus immigration of pre-adapted populations. In this paper, we analyze whether a host shift of the stolbur phytoplasma vector, *Hyalesthes obsoletus* from field bindweed to stinging nettle in its northern distribution range evolved sympatrically or by immigration. The new exploitation of stinging nettle has led to outbreaks of the grapevine disease bois noir caused by a stinging nettle-specific phytoplasma strain. Microsatellite data from populations from the northern and ancestral ranges provide strong evidence for sympatric host-race evolution in the northern range: Host-plant associated populations were significantly differentiated among syntopic sites ($0.054 < F_{HT} < 0.098$) and constant over five years. While gene flow was asymmetric from the old into the predicted new host race, which had significantly reduced genetic diversity, the genetic identity between syntopic host-race populations in the northern range was higher than between these populations and syntopic populations in ancestral ranges, where there was no evidence for genetic host races. Although immigration was detected in the northern field bindweed population, it cannot explain host-race diversification but suggests the introduction of a stinging nettle-specific phytoplasma strain by plant-unspecific vectors. The evolution of host races in the northern range has led to specific vector-based bois noir disease cycles.

Introduction

Inclusion of new hosts into the diet of phytophagous insects may have serious consequences for the distribution of pest species and epidemiological cycles (Bressan *et al.* 2007; Maixner *et al.* 2007). Since many phytophagous insects act as vectors of plant pathogens, changes in a vector's host use may influence the population structure of transmitted pathogens (Barrett *et al.* 2008; McCoy 2008). If the host range of an unspecialized vector becomes wider, pathogen interactions, including the possibility for genetic exchange between pathogen strains, may also increase (Christensen *et al.* 2005). Alternatively, restricting the host plant range may lead to specialized pathogen transmission cycles. Knowledge about changes in the population structure of vectors is therefore of great epidemiological importance. Adaptation to one host may cause mal-adaptation to another, leading to host-associated fitness trade-offs (Via 1990). These fitness trade-offs result in host shifts, rather than host-range expansions. Specialization and differentiation of host-plant affiliated populations may subsequently lead to genetically distinct host races (Diehl & Bush 1984; Dres & Mallet 2002).

Sets of populations are considered host races if four criteria are met: (1) they use different hosts, (2) they are sympatric, (3) they are genetically differentiated and (4) they exhibit appreciable (greater than 1 %) gene flow (Dres & Mallet 2002). Sympatric formation of host races is given when the host races are monophyletic and allopatric phases are very unlikely (Coyne & Orr 2004). In natural insect populations, extant host-race formation has often been linked with geographic range shifts (including new agricultural practices) of either the host plant or the insect, thus introducing new hosts (e.g. Diehl & Bush 1984 and references therein; Wingfield *et al.* 2011). Because geographic range shifts may be important in sympatric host-race evolution, a matter of debate is whether phases of allopatric divergence can be separated from true divergence in sympatry in non-island systems. Many cases reviewed by Dres and Mallet (2002) and Berlocher and Feder (2002) fulfill the criteria for evolution of host races in sympatry but an allopatric origin can usually not be ruled out (Coyne & Orr 2004). Feder *et al.* (2003) propose that allopatry could act in conjunction with sympatric host shifts to facilitate sympatric speciation by providing genetic material via secondary introgression.

Inclusion of completely new hosts is, however, not the only possible explanation for sympatric divergence. Alternatively, a potential host might go unused due to inapt conditions, e.g. temperature requirements, but become suitable if conditions improve (Singer *et al.* 1992). The latter is a likely scenario for many polyphagous species, which use a

range of hosts throughout their distribution, but are usually restricted to a few hosts tied in preference/acceptance hierarchies in different climatic or geographical region (Hodkinson 1997; Thomas *et al.* 2001). For genetic divergence to evolve in this scenario the populations should not merely exploit again a temporarily avoided host at the expense of the old host; population persistence on both hosts with differential utilization is required.

The xerothermic, polyphagous planthopper *Hyalesthes obsoletus* (Cixiidae) has been reported to feed on more than 50 plants but preferred host-plants for nymphal development are much fewer and differ among geographical regions. The major host plant of *H. obsoletus* is *Convolvulus arvensis* (field bindweed) but regional preferences include *Urtica dioica* (stinging nettle) in northern Italy (Alma *et al.* 1987; Lessio *et al.* 2007), *Lavandula angustifolia* (lavender) in southern France (Leclant & Lacote 1969; Sforza *et al.* 1999), and *Vitex agnus-castus* (monk's pepper) in Israel (Sharon *et al.* 2005). As a predominately Mediterranean species, *H. obsoletus* has its northernmost distribution range in southwest Germany and northern France (Alsace) where it is mainly found in climatically favorable river valleys associated with grapevine production. *Hyalesthes obsoletus* was until about 20 years ago a very rare species in Germany (e.g. Sergel 1986) associated with *C. arvensis*. Within the last two decades, however, it has become extremely common in suitable habitats. Its population growth is principally explained by the new utilization of *U. dioica* although a population increase on *C. arvensis* is also observed.

Hyalesthes obsoletus is the main vector of stolbur phytoplasma (16SrXII-A-group) (Lee *et al.* 1998), an emerging and major plant pathogen causing yellows diseases in grapevine (bois noir), maize (maize redness) lavender and various solanacious crops in Europe. Stolbur phytoplasma have obligate vector-based transmission and stolbur diseases are highly or completely determined by pathogen reservoirs in perennial herbaceous plants (Fos *et al.* 1992; Fialová *et al.* 2009; Kessler *et al.* 2011). Stolbur phytoplasma have two major strains, tuf-type-a and -b (Langer & Maixner 2004). *Urtica dioica* seems to be the only reservoir plant of tuf-type-a (Langer & Maixner 2004; Bressan *et al.* 2007). Tuf-type-b is associated predominantly with *C. arvensis* but is also found in other weedy plants other than *U. dioica.* Hence, being both nymphal host plant for *H. obsoletus* and reservoir plants of stolbur phytoplasma, differential use of *C. arvensis* and *U. dioica* by *H. obsoletus* may severely impact the epidemiology of stolbur diseases of agricultural crops by creating separate vector-based epidemiological cycles. In the case of the grapevine disease bois noir, the epidemiology is directly linked to these two herbaceous plants and not to grapevine, which is a dead-end host for the pathogen. The rapid spread of bois noir in central Western European

viticultural regions is primarily associated with the *U. dioica*-specialized tuf-type-a stolbur strain (Maixner *et al.* 2009).

Populations of *H. obsoletus* living on *U. dioica* and *C. arvensis* in Germany show morphological (Johannesen *et al.* 2008), behavioral (Grube 2010) and phenological differences (Lux *et al.* 2006). Transplant experiments have shown significantly reduced survival on alternative host plants (Kessler *et al.* 2011; Albert 2011). Despite these differences, both populations are fixed for an identical, derived mtDNA haplotype aa, which reached Germany via a westward range expansion north of the Alps from a historical source east of the Italian-Slovenian karst-devide (Johannesen *et al.* 2008; Figure 3). The genealogical identity of the two plant-associated populations indicate that the phenotypic differences evolved in sympatry since the shift to *U. dioica* within the past, perhaps, two decades. However, a secondary invasion of *H. obsoletus* west of the Alps via France has reached southern Germany, establishing a secondary contact zone there (Johannesen *et al.* 2012). This new lineage (haplotype bb) evolved in northern Italy where stinging nettle is a preferred plant. Although neither aa nor bb are associated with specific host-plants (Johannesen *et al.* 2008, 2012), indicating an innate ability of *H. obsoletus* to use both host plants, introduction of allopatric nuclear genetic variation via male-based gene flow might facilitate host-plant divergence.

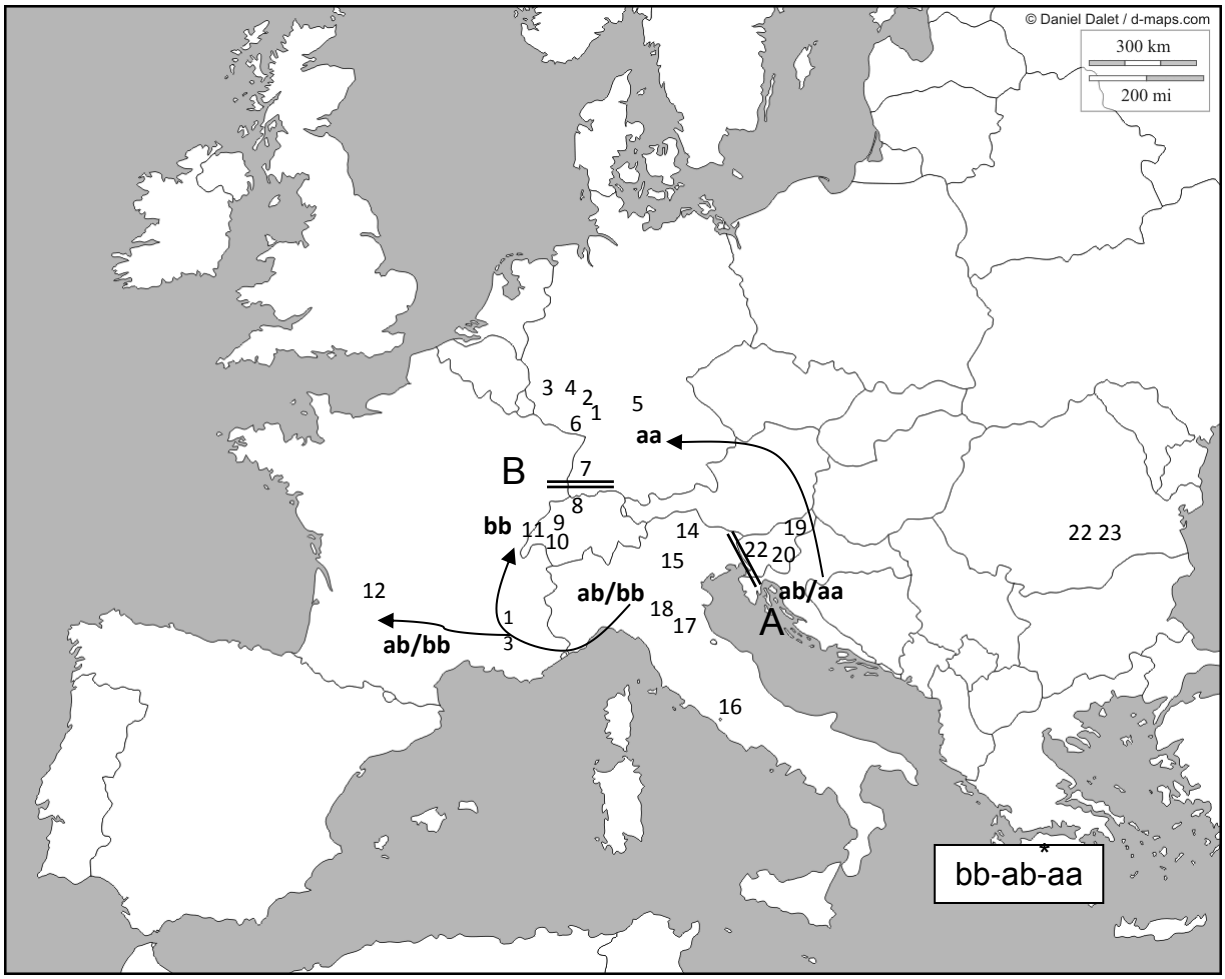

Figure 3 Map of Europe showing sampling sites of *H. obsoletus*, and the colonization history of Western Europe inferred from the distributions and genealogy (network, lower right) of the three signature mtDNA haplotypes, the ancestral ab (denoted by *) and the derived aa and bb (redrawn from Johannesen *et al.* 2008). Haplotype ab is found throughout southern Europe, from Slovenia (sites 20 - 22) to France (sites 12 - 13). Barrier A separates haplotypes aa and bb at the Italian-Slovenian karst-devide. Barrier B shows the secondary contact zone between aa and bb in the region of the German-Swiss border. Apart from admixture of haplotypes in the contact zone, German populations are monomorphic for aa whereas Swiss populations hold bb (not ab). Numbers refer to sites listed in Table 5. The sites Mayak (24, Russia) and Golan (25, Israel) are not shown on the map. Map from "d-maps.com kostenlose Karten", http://d-maps.com.

In this paper, we address the role sympatry and allopatry play in diversification in an open, non-island system and whether sympatric diversification is possible in a polyphagous insect with an innate capability to utilize both hosts. Specifically, in Germany we study whether the novel use of the common but formerly avoided plant *U. dioica* has resulted in a genetically divergent host population (host race) and, if affirmative, whether this took place

in sympatry or was caused by immigration of allopatrically *U. dioica*-adapted individuals from southern Europe. If local sympatric host-race formation occurred in Germany, we predict that 1) German *C. arvensis*- and *U. dioica*-associated populations are not only genetically divergent but also 2) that the *U. dioica*-associated population constitutes the derived host race and, consequently, the two plant-associated populations should 3) be relatively more closely related to each other than to other European populations. We expect the *U. dioica*-associated population to be the derived host race because *U. dioica* is described as new host plant in Germany and its use is associated with the emergence of *U. dioica*-tied stolbur phytoplasma tuf-type-a. The sympatric scenario is supported when 4) southern populations do not show host-related divergence. Alternatively, allopatric evolution would be indicated by the presence of two host races throughout the distribution range in Europe and/or by immigration of distinct *U. dioica*-associated populations from Italy where *U. dioica* is a preferred plant. In this scenario, we predict genetic similarity between German and southern populations from the same host plant and progressive founder events in *U. dioica*-associated populations towards the northern range edge.

To differentiate between sympatric vs. allopatric diversification process, we studied the genetic population structure of *H. obsoletus* in Europe at several hierarchical levels. Separate hierarchical analyses were done to reveal (1) the stability of host-plant association over time in a syntopic German population, (2) general host-plant association in Germany, (3) host-plant associations in phylogeographically ancestral regions in Italy and Slovenia, and (4) the genetic structure of *C. arvensis*- and *U. dioica*-associated populations in Western Europe outside Germany. To avoid a potential sampling bias affecting the interaction between host-plant association and geography, we analyzed the interaction by including (5) one syntopic sample site from each of four European regions. Finally, (6) the total genetic structure in Europe was analyzed (see Table 4 for a detailed explanation of the hierarchical analyses).

Table 4 Test hierarchies for the analyses of the genetic population structure of *H. obsoletus*. When a site was sampled in more than one year, only the latest sampling year was included in analysis (except level 1). *N* = number of population samples included in analysis, indicating the numbers of *C. arvensis*- and *U. dioica*-associated populations, respectively (level 1 - 5) and *C.a.* = *Convolvulus arvensis*; *U.d.* = *Urtica dioica*; *L.a.* = *Lavandula angustifolia*; *S.t.* = *Solanum tuberosum*; *V.a.* = *Vitex agnus-castus* (level 6). Samples included in each hierarchy are shown in Table 5.

Hierarchical analysis no.	Objective of analysis	Samples included	*N* (number of *C. arvensis*- and *U. dioica*-associated populations)
1	Consistency of host-plant affiliation over time in a syntopic German population	*C. arvensis*- and *U. dioica*-associated populations sampled over five consecutive years at the syntopic site Bacharach, Germany	5 + 5 = 10
2	General host-plant association in Germany	*C. arvensis*- and *U. dioica*-associated populations from all German sites (max. one host-sample per site, from the latest sampling year)	5 + 7 = 12
3	Host-plant association in the native region	*C. arvensis*- and *U. dioica*-associated populations from Italy and Slovenia	5 + 6 = 11
4	Genetic structure of *C. arvensis* and *U. dioica*-associated populations in Western Europe outside Germany	All non-German *C. arvensis*- and *U. dioica*-associated populations	7 + 10 = 17
5	Interaction between host-plant association and geography among European regions, omitting within-region genetic bias	One syntopic *C. arvensis*- and *U. dioica*-associated population from Germany, Switzerland, Italy, and Slovenia and one C. arvensis population from France	4 + 5 = 9
6	Total genetic structure in Europe	All available populations, including samples collected from additional host plants (max. one host-sample per site, from the latest sampling year)	12 (*C.a.*) + 17 (*U.d.*) + 1 (*L.a.*) + 3 (*S.t.*) +1 (*V.a.*) = 34

Material and Methods

Sample origin and scheme

Hyalesthes obsoletus were collected in seven European countries and in Israel (Figure 3). *Hyalesthes obsoletus* populations associated with the two focal host plants, *C. arvensis* and *U. dioica*, were sampled at 25 sites in a hierarchical manner with the aim of distinguishing between local and allopatric evolution of host-plant specificity (Table 5). The 25 sites included 42 population samples, which were defined as a sample of *H. obsoletus* collected from one site on one plant in one specific year. We collected population samples at five *C. arvensis* and *U. dioica* syntopic sites in Germany of which one site, Bacharach, was sampled over five consecutive years (2005 - 2009) to evaluate the constancy of host-plant affiliation. Four further syntopic sites were sampled in Switzerland, Italy, and Slovenia, and eight sites with exclusively *U. dioica* were obtained from Germany, Switzerland, and Italy. Finally, five populations sampled on other plants were included for comparison with *C. arvensis*- and *U.*

dioica-associated populations: *Lavandula angustifolia* (lavender) in France, *Solanum tuberosum* (potato) in Romania (two sites) and Russia and *Vitex agnus-castus* (monk's pepper) in Israel (Table 5). The specimens from Israel were sampled at several sites and therefore constitute more than one population. All specimens assigned to a population-sample were collected exclusively on a single plant species with an exhauster or with sweep nets in monoculture stands.

Table 5 Sample site data for *H. obsoletus* populations used for microsatellite analysis. Population numbers refer to sample sites in Figure 3. Syntopic sample sites with *C. arvensis* (*C.a.)* and *U. dioica* (*U.d.*) have the same number. The sites were sampled between 2004 and 2010; the sampling year appears in the population code. The hierarchical analysis numbers refer to population genetic test hierarchies explained in Table 4.

Population no. & code	Site	Country	Host plant	*N*	A_R	H_E	F_{IS}	Included in hierarchical analysis no.
1. D-B05-C	Bacharach	Germany	*C.a.*	22	4.161	0.742	0.154	1)
1. D-B06-C	Bacharach	Germany	*C.a.*	29	4.381	0.772	0.145	1)
1. D-B07-C	Bacharach	Germany	*C.a.*	20	4.637	0.801	0.119	1)
1. D-B08-C	Bacharach	Germany	*C.a.*	20	4.205	0.747	0.083	1)
1. D-B09-C	Bacharach	Germany	*C.a.*	20	4.514	0.794	0.028	1); 2); 5); 6)
1. D-B05-U	Bacharach	Germany	*U.d.*	27	3.678	0.703	0.069	1)
1. D-B06-U	Bacharach	Germany	*U.d.*	29	3.644	0.697	0.043	1)
1. D-B07-U	Bacharach	Germany	*U.d.*	20	3.396	0.673	0.064	1)
1. D-B08-U	Bacharach	Germany	*U.d.*	20	3.645	0.703	-0.041	1)
1. D-B09-U	Bacharach	Germany	*U.d.*	20	3.613	0.687	0.119	1); 2); 5); 6)
2. D-O07-C	Boppard	Germany	*C.a.*	7	4.058	0.725	0.073	2); 6)
2. D-O07-U	Boppard	Germany	*U.d.*	13	3.946	0.730	0.003	2); 6)
3. D-L05-C	Lieser	Germany	*C.a.*	18	4.275	0.757	0.021	2); 6)
3. D-L06-U	Lieser	Germany	*U.d.*	30	3.872	0.733	0.054	2); 6)
4. D-P08-C	Bernkastel-Kues	Germany	*C.a.*	22	4.123	0.744	0.098	2); 6)
4. D-P06-U	Bernkastel-Kues	Germany	*U.d.*	24	3.498	0.685	0.104	2); 6)
5. D-R06-U	Randersacker	Germany	*U.d.*	6	3.043	0.621	-0.056	2); 6)
6. D-U06-U	Ungstein	Germany	*U.d.*	30	3.550	0.690	-0.004	2); 6)
7. D-N09-C	Neuweier	Germany	*C.a.*	22	3.957	0.757	0.043	2); 6)
7. D-N09-U	Neuweier	Germany	*U.d.*	10	4.021	0.764	0.139	2); 6)
8. CH-A08-U	Arlesheim	Switzerland	*U.d.*	9	3.719	0.710	0.133	4); 6)
9. CH-L10-C	Le Landeron	Switzerland	*C.a.*	23	4.179	0.768	0.045	4); 5); 6)
9. CH-L10-U	Le Landeron	Switzerland	*U.d.*	24	4.100	0.757	0.091	4); 5); 6)
10. CH-M09-U	Morges	Switzerland	*U.d.*	10	4.140	0.781	0.079	4); 6)
11. CH-R08-U	Russin	Switzerland	*U.d.*	10	5.082	0.850	0.010	4); 6)
12. F-C09-C	Charentes	France	*C.a.*	22	4.512	0.782	0.097	4); 5); 6)

Population no. & code	Site	Country	Host plant	*N*	A_R	H_E	F_{IS}	Included in hierarchical analysis no.
13. F-H09-L	Mévouillon	France	*L.a.*	18	3.477	0.653	0.180	6)
14. I-E07-C	Eisacktal. Feldthurns	Italy	*C.a.*	15	3.505	0.660	0.102	3); 4); 5); 6)
14. I-E07-U	Eisacktal. Feldthurns	Italy	*U.d.*	10	3.905	0.739	0.158	3); 4); 5); 6)
15. I-C06-C	Treviso	Italy	*C.a.*	6	4.461	0.766	-0.027	3); 4); 6)
15. I-C06-U	Treviso	Italy	*U.d.*	6	4.833	0.814	0.084	3); 4); 6)
16. I-R06-U	Rom	Italy	*U.d.*	18	3.984	0.718	0.146	3); 4); 6)
17. I-M09-U	Modena	Italy	*U.d.*	16	4.393	0.764	0.063	3); 4); 6)
18. I-G09-C	Reggio Emilia	Italy	*C.a.*	8	4.487	0.770	0.111	3); 4); 6)
19. SLO-J04-U	Jareninski	Slovenia	*U.d.*	6	4.068	0.716	0.117	3); 4); 6)
20. SLO-K04-C	Krško	Slovenia	*C.a.*	7	4.474	0.802	0.159	3); 4); 6)
21. SLO-N06-C	Nova Gorica	Slovenia	*C.a.*	25	4.430	0.772	0.090	3); 4); 5); 6)
21. SLO-N06-U	Nova Gorica	Slovenia	*U.d.*	19	4.201	0.743	0.258	3); 4); 5); 6)
22. ROM-F08-S	Fundulea	Romania	*S.t.*	14	4.988	0.834	0.158	6)
23. ROM-R09-S	Radovanu	Romania	*S.t.*	14	5.106	0.850	0.212	6)
24. RUS-M09-S	Mayak	Russia	*S.t.*	20	5.047	0.834	0.154	6)
25. IL-G06-V	Golan	Israel	*V.a.*	20	5.744	0.888	0.212	6)

Sample size (*N*). Mean allelic richness (A_R) and mean expected heterozygosity (H_E) were calculated across seven loci while the inbreeding coefficient per population (F_{IS}) was estimated across six loci, excluding the sex-linked Locus C147. Estimates calculated with FSTAT and GENECLASS2. *C.a. = Convolvulus arvensis; U.d. = Urtica dioica; L.a. = Lavandula angustifolia; S.t. = Solanum tuberosum; V.a. = Vitex agnus-castus.*

Microsatellite and mtDNA procedure

Genomic DNA was extracted from whole specimens using the "High Pure PCR Template Preparation Kit" (Roche) following the manufacturer's protocol. We determined the DNA concentration using NanoDrop® (PEQLAB, Erlangen, Germany), and prepared working solutions (~30 ng/µl). Genetic variation was analyzed at seven microsatellite loci, B82, F56, F84, H120, E96, G85, C147 (Imo *et al.* 2011). Loci were amplified in two QIAGEN Multiplex PCR reactions with four and three fluorescent labeled primers, respectively (mix 1: B82, F56, F84 and H120, annealing temperature 60 °C; mix 2: E96, G85 and C147, annealing temperature 62.5 °C). For the multiplex PCR, we used a PCR volume of 10 µl (8.5 µl master mix and 1.5 µl DNA of *c.* 50 ng DNA per reaction). The master-mix contained a final concentration of 1x QIAGEN Multiplex PCR Master Mix, which provides 3 mM $MgCl_2$, and 0.2 µM of each primer. Cycling conditions were: 15 min at 95 °C, 30 cycles of 30 sec at 94 °C, 90 sec at 60/62.5 °C, 60 sec at 72 °C, followed by a

final extension of 30 min at 60 °C. We repeated samples that did not amplify directly up to two times. Samples were scored on an ABI 3130 capillary sequencer using 11.7 µl HiDi-formamide, 0.3 µl ROX 500 standard (Applied Biosystems, Carlsbad, USA) and 1 µl of the PCR product. Loci were genotyped using GENEMAPPER 4.0 (Applied Biosystems, Carlsbad, USA).

Genetic diversity

Genetic diversity of populations was estimated as expected heterozygosity (H_E), allele number (A) and allelic richness (A_R) (FSTAT 2.9.3, Goudet 1995). Differences in mean genetic diversity between *C. arvensis* and *U. dioica* populations were tested with t-tests (IBM SPSS Statistics 20.0). Estimates of expected heterozygosity were arcsin root transformed. Deviations from Hardy–Weinberg (*HW*) proportions and linkage disequilibrium (*LD*) between pairs of loci were analyzed for each population with the web-based version of GENEPOP (Raymond & Rousset 1995), using default settings. The sex-linked locus C147 was omitted in analyses of *HW* and *LD*. The presence of null alleles was evaluated with the program MICRO-CHECKER (van Oosterhout *et al.* 2004) using the first method of Brookfield (Brookfield 1996).

Population differentiation and assignment tests

Analysis of host plant affiliation and geographic location and their interaction on differentiation among populations was estimated using Bayesian clustering (STRUCTURE 2.3.3, Pritchard *et al.* 2000) and analyses of molecular variance (AMOVA) using ARLEQUIN 3.5 (Excoffier & Lischer 2010).

STRUCTURE was first used to estimate the most likely number of genetic clusters (K) for a set of individuals based on their multilocus genotypes. This analysis has no *a priori* hypothesis; rather it evaluates the global levels of differentiation that can be caused by multiple effects, for example host plant and/or geographic distance. In a second step, STRUCTURE was used to estimate the association of individuals to the previously determined number of clusters. Because Bayesian analysis may be influenced by relative genotype frequencies, e.g. if one set of populations is highly overrepresented in the data set, this may hide signals of clustering of other subsets. In our analysis, we therefore consciously included and excluded populations in six test-hierarchies that were designed to analyze plant specificity, and to distinguish between local host-race formation of *H. obsoletus* in Germany and immigration of *U. dioica*-adapted individuals from southern Europe. The hierarchies and their test objectives are described in Table 4.

For all STRUCTURE analyses, we used the admixture model and correlated allele frequencies between populations. These are considered the best settings when populations have similar frequencies due to migration or common ancestry, and therefore have low differentiation and subtle population structure (Falush *et al.* 2003). STRUCTURE was run with a burn-in and MCMC (Markov chain Monte Carlo) of 10,000 each. Twenty runs were carried out in order to quantify the amount of variation of the likelihood for each *K*. In order to infer the most likely number of genetic clusters, we compared the likelihood plateau found by STRUCTURE and the method developed by Evanno *et al.* (2005). According to Evanno *et al.* (2005), the distribution of *Ln P(D)* in the STRUCTURE output does not always show a clear mode for the true *K*, but an ad hoc quantity based on the second rate of change of the likelihood function with respect to *K* (ΔK) does usually show a clear peak at the true value of K, except for $K = 1$, which cannot be detected by this method. The distribution of *Ln P(D)* and ΔK were compared for each test hierarchy. If the two methods contradicted, both possible numbers of genetic clusters were considered for further analyses. For each optimal *K*, we checked whether the population membership assignments were constant among the 20 runs. As this was the case, assignment percentages varied by 0 – 1 % (results not shown), each bar chart presented in the Results was drawn from the result of a single run.

We performed hierarchical analyses of molecular variance (AMOVA) with ARLEQUIN (Excoffier & Lischer 2010) to estimate the level of genetic differentiation between host plant and geographically affiliated populations. This method apportions genetic variation within and among pre-defined groups and allows quantifying how much of the overall genetic variation can be assigned to the different genetic groups, as revealed with STRUCTURE. We therefore performed AMOVA for each hierarchical level of the STRUCTURE analysis.

The genetic identity among *H. obsoletus* populations associated with *C. arvensis* and *U. dioica* was estimated with a maximum likelihood phenogram calculated with the program PHYLIP 3.69 (Felsenstein 2010). The phylogenetically divergent *V. agnus-castus* population in Israel (Johannesen *et al.* 2008) was used as outgroup.

We used assignment tests to infer signals of relatedness (ancestry) between the two German host-plant populations and relative to putative STRUCTURE populations in Italy, Slovenia and Switzerland. These tests differed from the STRUCTURE analyses in that individuals of each focal host-race population were "forced assigned" to a reference population, which was defined for each individual as the reference population with the

highest rank score. For this analysis, all population samples within each of the five regional categories were pooled to give a single regional sample. The assignments were performed with GENECLASS2 (Piry *et al.* 2004) using the algorithm of Rannala and Mountain (1997). The number of individuals in each German host-plant population that were assigned by first rank to one of the four reference populations were compared between the two German host-plant populations with Fisher's exact tests.

Results

Genotypes were obtained for 729 individuals from 25 sites in Europe and Israel. All loci were polymorphic across all populations with between 16 (F84) and 28 (E96) (mean 21) alleles per locus. Out of a total of 148 observed alleles, 20 (13.5 %) were private, occurring in one population only. All private alleles occurred at low frequencies (0.020 - 0.111). The highest number of private alleles (10) was recorded in the Israel population, which constitutes a separate phylogeographic lineage (Johannesen *et al.* 2008). The European populations each contained two or less private alleles. No significant linkage disequilibrium was detected for any locus pair in any population after Bonferroni correction (Rice 1989).

Genetic diversity and HWE

The mean expected heterozygosity per population ranged from 0.621 to 0.888 (Table 5). Excluding the sex-linked locus C147, the mean inbreeding coefficient was $F_{IS} = 0.070$ ($p < 0.001$). Significant deviations from Hardy Weinberg at the 0.05 level was recorded in 9 (3.3 %) out of 276 possible single locus exact tests after Bonferroni correction. (For the sex-linked locus C147, only females were included in the locus-specific analysis). Locus G85 showed homozygote excess in 6 of 42 population samples. This could have been caused by the possible presence of null alleles in these populations. Mean null allele frequency over all loci and populations was 0.02, the highest mean null allele frequency was found in locus G85 with 0.09 and in the French population on lavender with 0.08. These low frequencies should not bias population genetic analyses. No population showed an overall deviation from Hardy-Weinberg equilibrium.

Mean expected heterozygosity per sample was independent of sample size within each geographic region ($0.009 < R^2 < 0.07$, $p > 0.05$) and among all samples ($R^2 = 0.02$, $p > 0.05$), thus tests considering H_E were based on the un-weighted expected estimates. Genetic diversity was lost during the shift of *H. obsoletus* from *C. arvensis* to *U. dioica* in

Germany: Mean expected heterozygosity and mean allelic richness was significantly lower in German *U. dioica*- than in German *C. arvensis*-associated populations (H_E: 0.699 vs. 0.760, T = -4.274, df = 18, $p < 0.001$; A_R: 3.6 vs. 4.3, T = -5.602, df = 18, $p < 0.001$). Of the 100 alleles found in German populations, 22 alleles were found exclusively in *C. arvensis*- and 3 alleles found exclusively in *U. dioica*-associated *H. obsoletus*, which indicated that about 20 % of the alleles were lost in the individuals that switched hosts. In total, 38 alleles found in German populations differed significantly (t-test, df = 18, $p < 0.05$) in frequency between *H. obsoletus* collected from *C. arvensis* and *U. dioica*.

Populations in the ancestral ranges Italy and Slovenia did not differ significantly in genetic diversity and were pooled for comparative tests with German and Swiss populations. Host plant: *C. arvensis* vs. *U. dioica* in Italy: H_E: T = -0.684, df = 5, p = 0.524; A_R: T = -0.345, df = 5, p = 0.744. *C. arvensis* vs. *U. dioica* in Slovenia: H_E: T = -0.424, df = 9, p = 0.720; A_R: T = -0.289, df = 9, p = 0.779.

Genetic diversity in German *U. dioica*-associated populations was also significantly reduced relative to populations in the ancestral ranges Italy and Slovenia (i.e. regions with haplotypes ab) (H_E: 0.699 vs. 0.751, T = -3.105, df = 20, p = 0.006; A_R: 3.6 vs. 4.2, T = -4.539, df = 20, $p < 0.001$), and relative to Swiss populations situated south of the secondary contact zone (region with haplotypes bb but not ab) (H_E: 0.699 vs. 0.775, T = -2.872, df = 11, p = 0.017; A_R: 3.6 vs. 4.3, T = -2.882, df = 11, p = 0.016). By contrast, genetic diversity of German *C. arvensis*-associated populations did not differ significantly from Italian/Slovenian populations and from Swiss populations (ANOVA: H_E: $F_{2,19}$ = 0.662, p = 0.572; A_R: $F_{2,19}$ = 0.108, p = 0.898).

The Italian and Slovenian populations were pooled in the above analyses as neither host plant populations (*C. arvensis* vs. *U. dioica* in Italy: H_E: T = -0.684, df = 5, p = 0.524; A_R: T = -0.345, df = 5, p = 0.744; *C. arvensis* vs. *U. dioica* in Slovenia: H_E: T = -0.424, df = 2, p = 0.713; A_R: T = -0.478, df = 2, p = 0.680) nor regional populations (Italy vs. Slovenia: H_E: T = -0.370, df = 9, p = 0.720; A_R: T = -0.289, df = 9, p = 0.779) differed significantly in genetic variability.

Population differentiation

Level 1). We tested the consistency of host-plant affiliation over time in a syntopic German population, Bacharach, which had been sampled over five consecutive years (2005 - 2009). STRUCTURE analysis and ΔK calculations (Evanno *et al.* 2005) of *C. arvensis*- and *U. dioica*-associated populations, clearly inferred two genetic clusters, $\Delta K(2)$ = 62.5

(Figure 4a). The percentage of membership for *C. arvensis*-associated populations assigned to cluster 1 was 75 % to 89 % per year, and 82 % to 92 % per year for *U. dioica*-associated population assigned to cluster 2 per year (Figure 5a).

Host-plant-associated genetic differentiation was highly significant, $F_{HT} = 0.095$, $p < 0.01$, and accounted for 97 % of the total variance at this syntopic locality ($F_{HT}/F_{ST} = 0.095/0.098 = 0.97$). The variance explained by sample year within host-associations was not significant ($F_{SH} = 0.003$, $p = 0.296$; Table 6a).

Level 2). We tested the general host-plant association in Germany by analyzing all German populations. To avoid overrepresentation of single sites, we included only samples from the latest sample year from each site. Two genetic clusters, $\Delta K(2) = 54.0$ (Figure 4b) were inferred. For all populations except the southernmost population Neuweier, the percentage of membership of *C. arvensis*-associated populations to cluster 1 was 80 – 90 %, and the membership of *U. dioica*-associated populations to cluster 2 was 69 – 91 %. The *C. arvensis* and *U. dioica*-associated populations from Neuweier could not be assigned unambiguously; the *C. arvensis*-associated population had a membership of 64 % to cluster 1, while the membership percentage of the *U. dioica*-associated population to cluster 2 was 51 % (Figure 5b). Host-plant-associated genetic differentiation was highly significant in Germany, $F_{HT} = 0.054$, $p < 0.001$, and accounted for 65 % of the total variance, $F_{ST} = 0.083$, $p < 0.001$. Genetic variance among populations within host associations explained 35 % of the total variance ($F_{SH} = 0.030$, $p < 0.001$; Table 6b). Within German *C. arvensis*-associated populations, $F_{ST} = 0.032$ ($p < 0.001$) and within German *U. dioica*-associated populations, $F_{ST} = 0.024$ ($p < 0.05$). Single locus differentiation estimates are shown in Appendix Table 7a.

Level 3) To infer host-plant associations of *H. obsoletus* in the native region we analyzed populations from Italy and Slovenia, $N = 11$. One genetic cluster was inferred for the Italian and Slovenian *C. arvensis*- and *U. dioica*-associated populations. The plateau of *Ln P*(*D*) was reached at $K = 1$. ΔK was extremely low, $\Delta K \leq 1.1$, and heterogeneous for all *K*'s (Figure 4c). AMOVA analysis failed to find host-plant genetic variance, $F_{HT} = 0.003$, whereas there was significant geography-based genetic variance within host plant populations, $F_{SH} = 0.048$ ($p < 0.001$; Table 6c).

Level 4) STRUCTURE analysis and ΔK calculations for all non-German populations associated with *C. arvensis* and *U. dioica* in Western Europe, $N = 17$, inferred two genetic clusters, $\Delta K(2) = 56.0$ (Figure 4d), which were associated with geographic range, not host

plant. Cluster 1 consisted of the populations from France and Switzerland (73 – 96 % membership assignment), whereas cluster 2 consisted of Italian and Slovenian samples (69 - 97 % membership assignment). Total genetic differentiation was $F_{ST} = 0.077$ ($p < 0.001$). When populations were grouped according to the clusters inferred by STRUCTURE, differentiation between the regions was low, but significant, $F_{RT} = 0.028$ ($p < 0.001$; Table 6e), explaining 36 % of the total genetic variance. Genetic differentiation was not significant when populations where grouped relative to host plant, $F_{HT} = -0.002$ (Table 6d). Single locus differentiation estimates are shown in Appendix Table 7b.

Level 5). To test the interaction between host-plant association and geography among European regions while ruling out the within-region associated variance contributions to the differentiation estimate, we analyzed syntopic sites, i.e. single sites containing *H. obsoletus* sampled on *C. arvensis* and *U. dioica*, respectively, from one syntopic site in Germany, Switzerland, Italy, and Slovenia. We did not obtain samples from *U. dioica*-associated *H. obsoletus* from southern France and therefore only included French *C. arvensis*-associated samples. Cluster analysis performed on geographically separated syntopic *C. arvensis*- and *U. dioica*-associated populations inferred three genetic clusters for STRUCTURE *Ln P(D)* and ΔK (3) = 13.2 (Figure 4e). The populations clustered according to geography, not host plant. One cluster consisted of the two German populations (76 and 87 % membership assignment) and the French *C. arvensis*-associated population (64 % membership assignment), the second cluster included the two Swiss populations (84 and 85 % membership assignment) and the third cluster was made up of the two Italian (68 and 93 % membership assignment) and the two Slovenian (69 and 75 % membership assignment) populations (Figure 5c). The geographic clusters revealed by STRUCTURE accounted for 38 % of the total genetic variance ($F_{ST} = 0.085$, $p < 0.001$; $F_{RT} = 0.032$, $p = 0.002$; Table 6g). Host-plant affiliation did not contribute to the genetic variance, $F_{HT} = -0.002$ ($p > 0.05$; Table 6f)

Level 6) A final analysis was conducted with all available populations. The inferred number of clusters differed between STRUCTURE's *Ln P(D)* and Evanno *et al.* (2005) ΔK estimation methods. ΔK inferred two clusters, $\Delta K(2) = 40.9$, but a second, much smaller peak was found at $\Delta K(6) = 6.2$. Ln P(D) indicated that $K = 6$ was most likely (Figure 4f). When two clusters were assumed, all German *U. dioica*-associated populations, the southern German *C. arvensis*-associated population Neuweier and three Swiss populations from *C. arvensis* and *U. dioica* (Le Landeron and Arlesheim) were assigned to cluster 1 (membership assignment 73 % - 96 %) while one German *C. arvensis*-associated population (Boppard),

the populations from France, Italy, Slovenia, Romania, Russia, Israel and one south-west Swiss *U. dioica*-associated population (Russin) were assigned to cluster 2 (membership assignment 65 % - 94 %). The remaining populations consisting of German *C. arvensis*- and one Swiss *U. dioica*-associated population had an intermediate assignment of individuals (membership assignment 42 % - 58 % belonging to either cluster) (Figure 5d). An AMOVA was run with these two clusters, omitting intermediate populations. In this analysis, 33 % of the total genetic variance was caused by differentiation between the two clusters (F_{RT} = 0.036, p < 0.001; F_{ST} = 0.109, p < 0.001; Table 6h). When six clusters were assumed, the clustering of the populations almost perfectly matched the pattern found by the maximum likelihood phenogram (Figure 6; Table 8 Appendix). Both analyses clearly separated the German *C. arvensis* from the German *U. dioica* samples. The Swiss populations as well as the *C. arvensis* population from Neuweier, Germany, were intermediate and dispersed between different clusters. Italian and Slovenian populations clustered together. The population from French lavender strongly differed from all other populations. An AMOVA with the six clusters inferred by STRUCTURE revealed that 38 % of the total genetic variance was caused by differentiation between these clusters (F_{HT} = 0.038, p < 0.001; F_{ST} = 0.099, p < 0.001) (Table 6i). A table with all pair-wise F_{ST} estimates is presented in Appendix Table 9.

Assignment tests

The assignment tests are presented in Figure 7. The percentage of German *U. dioica*-associated individuals assigned with first rank to the German *C. arvensis* population (61 %, mean assignment score 0.86) was significantly higher (p < 0.001) than the percentage of German *C. arvensis*-associated individuals assigned with first rank to the German *U. dioica*-associated population (27 %, mean assignment score 0.86). By contrast, the percentage of German *C. arvensis*-associated individuals assigned with first rank to the Swiss and the Slovenian population were significantly higher than the percentages of German *U. dioica*-associated individuals (Switzerland: 48 %, p = 0.017, mean assignment score 0.77; Slovenia: 16 %, p < 0.001, mean assignment score 0.72). As a group of individuals, the German *U. dioica*-associated population was assigned with 100 % to the German *C. arvensis*-associated population whereas the German *C. arvensis*-associated population as a group was assigned with 100 % to the Swiss population.

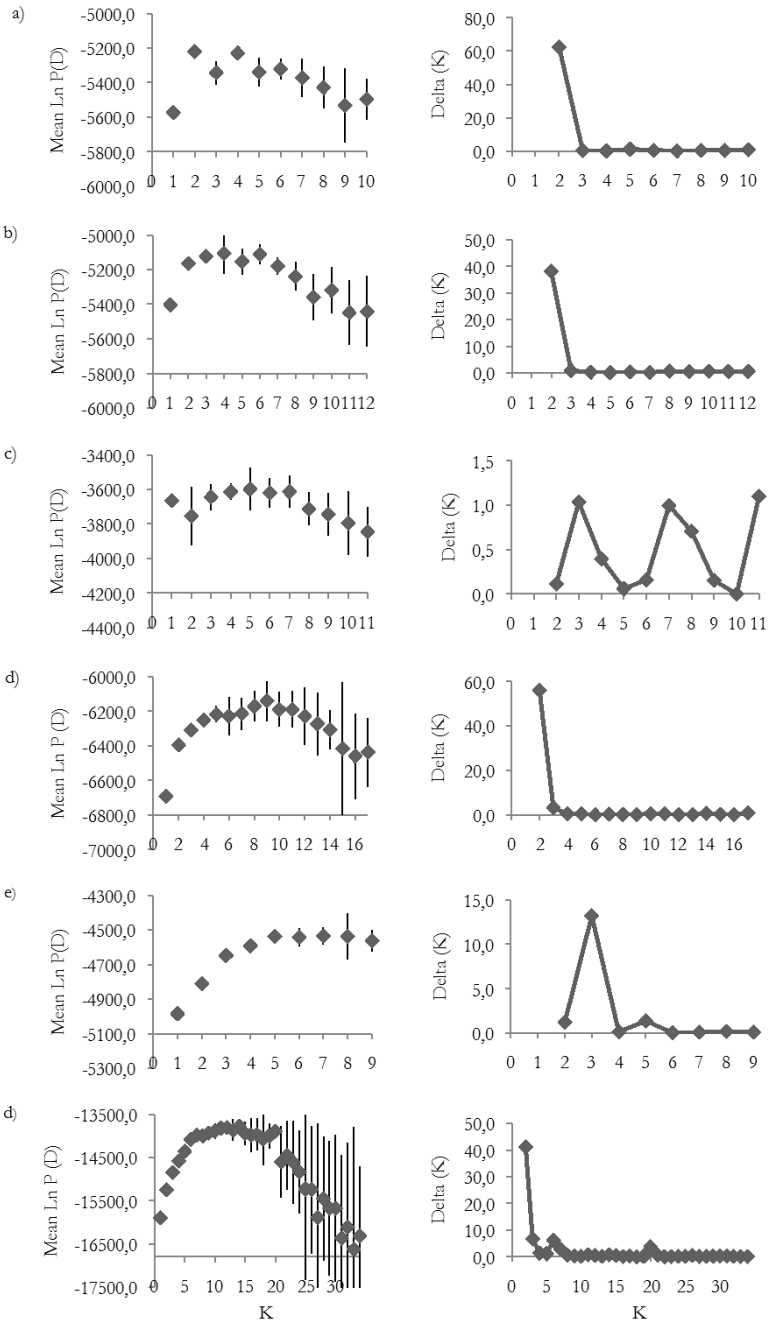

Figure 4 STRUCTURE analysis of hierarchical level no. 1 to 6 (a-f). In the STRUCTURE output (mean *Ln P*(*D*) over 20 runs), left column, the most likely number of genetic clusters (*K*) is indicated by the beginning of the plateau phase of *Ln P*(*D*). In the ad hoc quantity based on the second rate of change of the likelihood function with respect to *K* (Δ*K*) (Evanno *et al.* 2005), right column, a peak indicates the most likely *K*. Please note the different scaling.

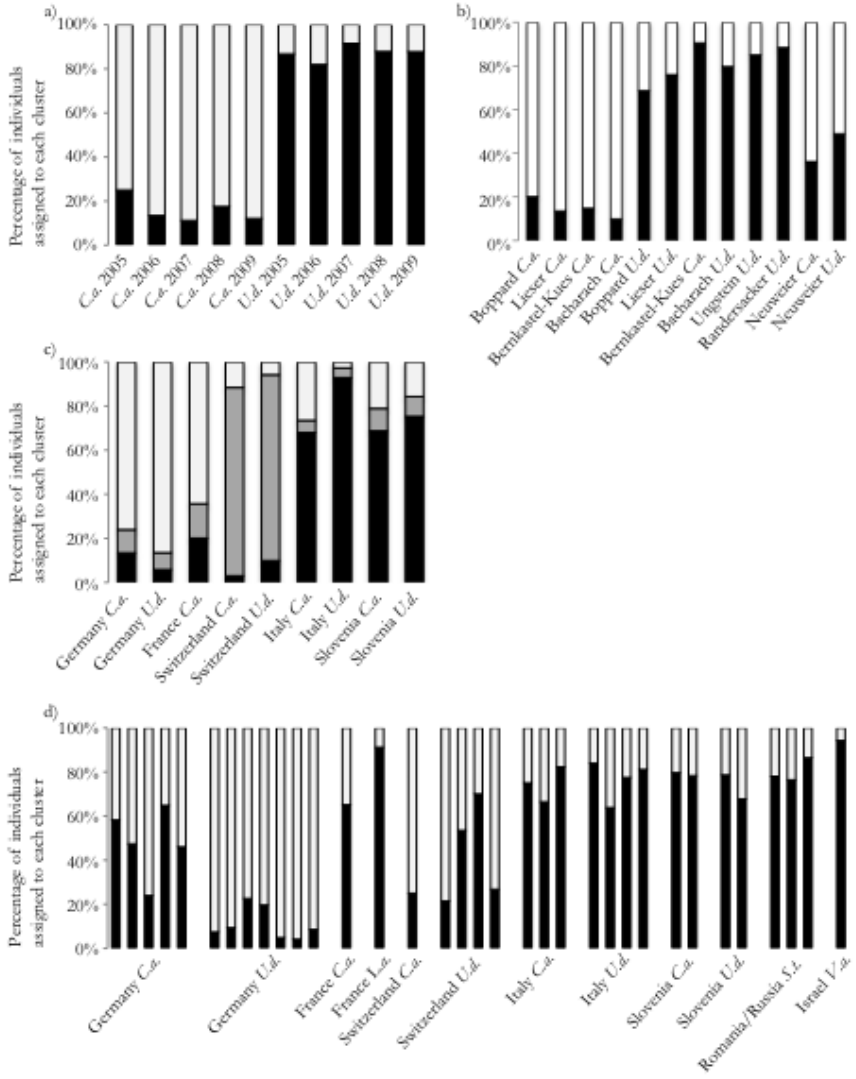

Figure 5 Bar charts showing population membership assignments in hierarchical STRUCTURE analyses. a) Level 1: Populations from Bacharach, Germany, sampled over five consecutive years from *C. arvensis* and *U. dioica*. In *C. arvensis*-associated populations, membership assignments to cluster one (white) were 75 – 89 % while the assignment of *U. dioica*-associated populations to cluster two (black) was 82 – 92 %. b) Level 2: All German populations (only latest year of each site). Except for the intermediate, southernmost population, Neuweier, *C. arvensis*-associated populations had assignments of 80 – 90 % to cluster one (white), *U. dioica*-associated populations had 69 – 91 % assignments to cluster two (black). c) Level 5: One syntopic site (*C. arvensis* and *U. dioica*) per country. Populations cluster according to geography, Germany (white), Switzerland (grey), Italy/Slovenia (black) but not host-plant affiliation. d) Level 6: All sampled populations showing German *U. dioica* populations differing the most. *C.a. = Convolvulus arvensis; U.d. = Urtica dioica; L.a. = Lavandula angustifolia; S.t. = Solanum tuberosum; V.a. = Vitex agnus-castus.*

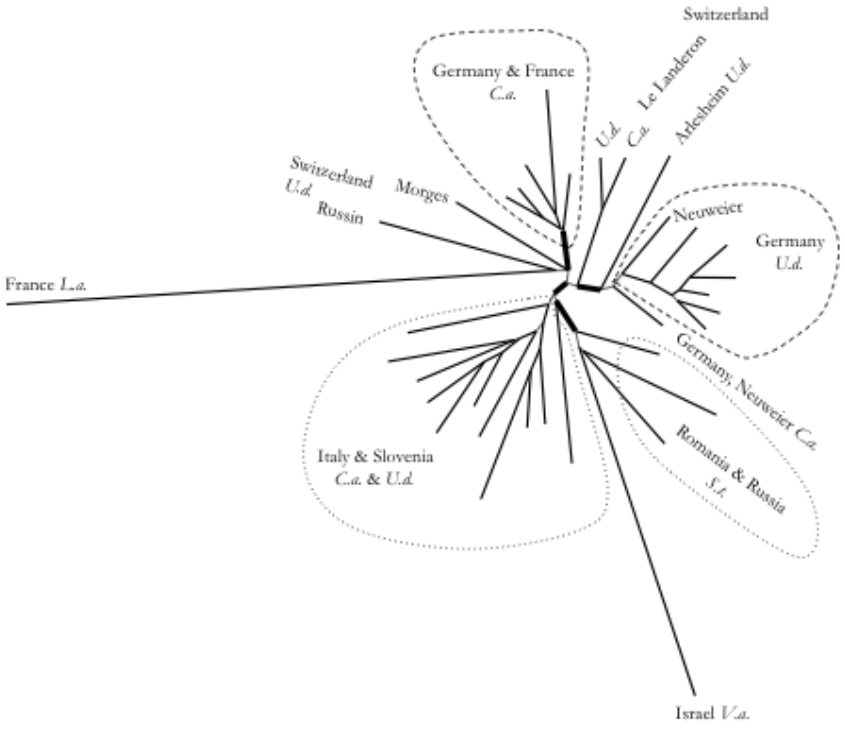

Figure 6 Maximum likelihood tree based on allele frequencies calculated with seven microsatellite loci for all available populations of *H. obsoletus*. Significant inner branches are shown with bold lines. *C.a.* = *Convolvulus arvensis; U.d.* = *Urtica dioica; L.a.* = *Lavandula angustifolia; S.t.* = *Solanum tuberosum; V.a.* = *Vitex agnus-castus.*

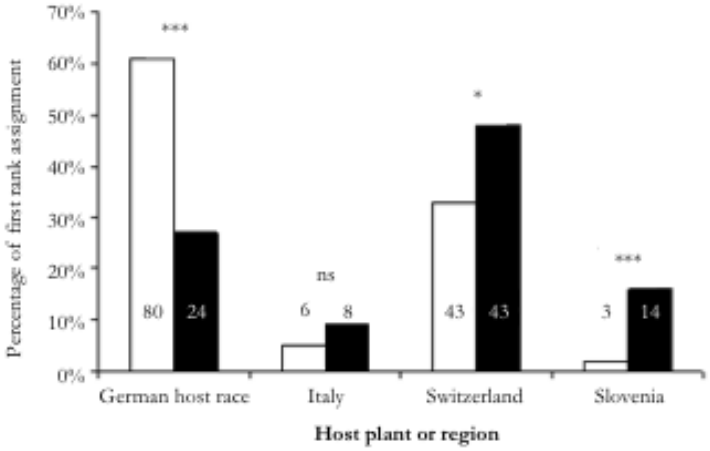

Figure 7 Percent German host-race individuals assigned with first rank to reference populations. White bars show the percentage of German *U. dioica*-associated individuals assigned to the German *C. arvensis*-associated population and three countries of putative origin. Black bars show the percentage of German *C. arvensis*-associated individuals assigned to the *U. dioica*-associated population and three countries of putative origin. Numbers indicate the number of observations. $*p < 0.05$; $***p < 0.001$, ns = not significant (Fisher's exact tests).

Table 6 Hierarchical *F*-statistics over all microsatellite loci. It is shown to which extent total among-population differentiation (F_{ST}) can be assigned to host-plant (F_{HT}) and geographic variance (F_{SH}) within host-plant associations, or to geographic variance among (F_{RT}) and within regions (F_{SR}). Significant F_{HT} values indicate restricted gene flow among *H. obsoletus* populations infesting *C. arvensis* and *U. dioica*. Populations included in the analyses are shown in Table 5.

Type of analysis	F_{ST}	F_{SH} or F_{SR}	F_{HT} or F_{RT}
(a) *H. obsoletus* from Bacharach, Germany, collected over five consecutive years and grouped according to host-plant affiliation (F_H, hierarchical analysis no. 1)	0.098***	0.003	0.095**
(b) German *H. obsoletus* populations, grouped according to host-plant affiliation (F_H, hierarchical analysis no. 2)	0.083***	0.030***	0.054***
(c) *H. obsoletus* from the native region Italy and Slovenia, grouped according to host-plant affiliation (F_H, hierarchical analysis no. 3)	0.050***	0.048***	0.003
(d) European non-German *H. obsoletus* populations from *C. arvensis* and *U. dioica*, grouped according to host-plant affiliation (F_H, hierarchical analysis no. 4)	0.064***	0.066***	-0.002
(e) European non-German *H. obsoletus* populations from *C. arvensis* and *U. dioica*, grouped according to regional affiliation, as classified by Structure (F_R, hierarchical analysis no. 4)	0.077***	0.051***	0.028***
(f) One *C. arvensis*- and one *U. dioica*-associated population from Germany, France, Switzerland, Italy, and Slovenia, grouped according to host-plant affiliation (hierarchical analysis no. 5)	0.076***	0.078***	-0.002
(g) One *C. arvensis*- and one *U. dioica*-associated population each from Germany, France, Switzerland, Italy, and Slovenia, grouped according regional affiliation, as classified by STRUCTURE (F_R, hierarchical analysis no. 5)	0.085***	0.054***	0.032**
(h) All *H. obsoletus* populations, grouped according to regional affiliation (2 clusters), as classified by STRUCTURE (F_R, hierarchical analysis no. 6)	0.109***	0.076***	0.036***
(i) All *H. obsoletus* populations, grouped according to host and regional affiliation (6 clusters), as classified by STRUCTURE (F_H, hierarchical analysis no. 6)	0.099***	0.064***	0.038***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. F_H = Host plant analysis. F_R = Geographic analysis.

Discussion

We found strong evidence for genetic host races of the polyphagous *H. obsoletus* associated with *C. arvensis* and *U. dioica* in mid-west Germany (Figure 5). Host-plant affiliated differentiation in mid-west Germany was verified at four syntopic locations and over five consecutive years at one syntopic site. For *H. obsoletus* in mid-west Germany, three of the four criteria for host races proposed by Dres and Mallet (2002) are met: the use of different hosts, sympatry, and genetic differentiation. The fourth criterion, exhibition of some gene flow, is most likely met, but is not of importance here, since it is merely needed to differentiate between host races and sister species. We therefore consider the populations of *H. obsoletus* associated with different host plants to have reached host race status in Germany.

Formation of host-races in mid-west Germany can be explained by two principle processes: 1) by local, sympatric host-shift with specialization on the newly preferred host

U. dioica or, 2) by recent immigration of *U. dioica*-adapted individuals from southern Europe where *U. dioica* is a common host (Alma *et al.* 1987; Lessio *et al.* 2007). Confirmation of sympatric evolution requires a monophyletic origin of the derived population relative to the sympatric ancestral population without allopatric phases (Coyne and Orr 2004). Confirmation is most easily made in island systems (Barluenga *et al.* 2006) or when populations suddenly evolve on newly introduced resources (Feder *et al.* 1990). Detecting sympatric divergence on common co-occurring resources in open non-island systems is more elusive. In this study, we conceived a range of tests to assess the plausibility in the latter setting between plant-associated populations of the planthopper *H. obsoletus*.

The nuclear data collected in this study support mtDNA haplotype distributions and provide evidence for sympatric host-race evolution but also suggests signs of immigration, which is most likely unrelated to host-race genetic divergence. Sympatric host-race evolution was supported by lack of host races at syntopic sites in the ancestral, allopatric ranges in Italy and Slovenia. Despite relatively high genetic divergence between the host races in Germany, a monophyletic origin was indicated at nuclear loci by the two populations being closely related when comparing syntopic populations in Europe (Level 5 analysis, Figure 5c). In accordance with our test requirements, the German *C. arvensis*-associated population was the inferred source of the derived *U. dioica*-associated population, the latter being more related to the former than vice versa. *Urtica dioica*-associated populations also had significantly reduced genetic diversity, which is more likely explained by a founder effect during the host shift than by stepping stone immigration because there was no sign of reduced diversity in *U. dioica* or *C. arvensis*-associated populations at or south of the secondary contact zone. Finally, the data imply that the host shift was a single event (contrary to multiple shifts) because diversification is affecting all seven loci equally (Appendix Table 7), suggesting genetic drift as the diversifying force on these loci. These findings combined support local sympatric diversification against the immigration hypothesis.

Despite sympatric divergence, the assignment tests assigned German *C. arvensis*-associated populations to Swiss populations more than to German *U. dioica*-associated populations. This signal may partly result from different levels of genetic variability, the latter population being depauperate, but it also implies male-biased genetic exchange. The latter would explain the intermediate position of Swiss populations in several analyses, and why the phylogeographically young German *C. arvensis*-associated populations have slightly, albeit not significantly, more nuclear genetic variability than southern ancestral populations.

Although introgression of nuclear genetic diversity may have introduced allopatric genetic variance for sympatric host-race evolution in Germany, both the lack of host-related genetic divergence (at these loci) in the ancestral ranges of both mtDNA lineages and admixture traced in the original host (*C. arvensis*) rather than in the new host (*U. dioica*) in Germany suggest that allopatry did not contribute significantly to the host-race process. Instead, this pattern implies immigration of non-diverged (plant unspecialized) *H. obsoletus*. Immigration is able to explain the co-ancestry and recent emergence of the *U. dioica*-associated stolbur strain tuf-a in Germany, Switzerland and Northeast France (Johannesen *et al.* 2012) across the contact zone by transfer via unspecific vectors into the *U. dioica*-host-race cycle. The evolution of host races has now created two vector-based disease cycles of bois noir in the northern distribution range.

How and why does host race formation in a polyphagous insect evolve in the presence of two abundant and in many regions co-occurring host plants? The data for the polyphagous *H. obsoletus* agree with two general findings observed in sympatric diversification of monophagous insects, findings that likely apply to resource limited polyphagous species as well. First, most host races display allochronic isolation (e.g. Horner *et al.* 1999; Groman & Pellmyr 2000; Diegisser *et al.* 2006; Dambroski & Feder 2007). The host races of *H. obsoletus* in Germany display only 1 - 2 weeks overlap in flight activity (Maixner & Langer 2006) while the overlap between genetically non-divergent populations in Italy is longer, 3 - 4 weeks (Forte *et al.* 2010). Slight changes in flight period may induce host-associated genetic divergence rapidly, as observed in the widespread fleahopper *Pseudatomoscelis seriatus* where divergence was found in allochronic but not in temporal overlapping populations (Barman *et al.* 2012). Second, changes in host use are often observed at the species' geographic range borders (e.g. Hodkinson 1997; Thomas *et al.* 2001; Diegisser *et al.* 2009; Zuber & Widmer 2009), where climate conditions are more extreme and/or the distributions of the preferred hosts differ from the center of the distribution range. Mid-western Germany is the northern distribution range limit of *H. obsoletus*. Even though *U. dioica* is not scarce in this region, the plant-growing season is shorter and winter temperatures are lower than in more southern regions. *Hyalesthes obsoletus* associated with *U. dioica* requires a higher day degree sum for completion of larval development, but increasing mean temperatures within the last 50 years (Maixner & Hoffmann 2011), especially in spring and fall, has led to longer growing periods for all plants (Menzel 2000), including *U. dioica*. The extended nymphal developmental period on *U. dioica* (Cargnus *et al.* 2012) might therefore be compensated by a longer feeding period (earlier start in spring and/or later end in fall). This may explain why it is now possible for

H. obsoletus in mid-west Germany to develop on *U. dioica* in the microclimatically less suitable plains. Additionally, rising mean winter temperatures may allow survival of nymphs on *U. dioica* roots, which reach depths of only 15 cm versus 25 cm for *C. arvensis*.

Hyalesthes obsoletus likely possesses the innate capability to utilize both *C. arvensis* and *U. dioica*, as indicated by plant-independent mtDNA haplotype distributions (Johannesen *et al.* 2008). However, it probably exhibits preference/acceptance hierarchies, as has been shown in many other polyphagous insects (Singer *et al.* 1992). Rank order of preference may differ strongly within and between populations, which can lead those individuals with the same host to meet and mate on their preferred host (Johnson *et al.* 1996; Rice 1984; Singer *et al.* 1989). As mate choice is often coupled with habitat choice, assortative mating can arise as a by-product, resulting in reproductive isolation (Dieckmann & Doebeli 1999). On the other hand, change in preference is not necessarily a prerequisite for a host shift (Singer 1982). Depending on the availability and suitability of the hosts at any given time, hosts lower on the rank order of preference may be used if higher-ranking hosts are not available (Courtney *et al.* 1989; Jaenike 1990; Pilson & Rausher 1988). New populations can thus be founded on novel host species without changes in the preference characteristics of the adults (Singer 1982).

Factors that facilitate the formation of host races include improved nutrition (Blair *et al.* 2010), enemy-free space (Denno *et al.* 1990; Gross & Price 1988) and reduction of intra-specific and inter-specific competition (Feder *et al.* 1995; Messina 2004). Host shift and subsequent adaptation to a novel host usually leads to genetic trade-offs and/or costs. In the polyphagous pea aphid complex *Acyrthosiphon pisum*, genetic trade-offs prevent the optimal use of multiple host plants for biotypes (Peccoud & Simon 2010). For *H. obsoletus,* the costs of switching to the initially less suitable host *U. dioica* could be related to different nutritional profiles, as is supported by survival and longevity experiments (Kessler *et al.* 2011; Albert 2011). An interesting notion that has been put forward to explain adaptation in tri-trophic systems is the role of bacterial endosymbionts. Phytoplasmas have been shown to render plants more suitable for phloem-feeders or even convert non-hosts into hosts for phloem-feeding insects (reviewed in Hogenhout *et al.* 2008). The novel use of *U. dioica* in Germany coincides with the emergence of the stolbur strain tuf-type-a, a recent immigrant (J. Johannesen *et al.* 2012). Because stolbur infection apparently does not affect nymphal growth rates (Kaul *et al.* 2009) and adult survival (Albert 2011), but may affect plant attraction (chapter 3), the possibility for a positive (or manipulative) stolbur effect on *H. obsoletus'* ability to exploit infected *U. dioica* remains suggestive (see e.g. Mayer *et al.* 2008)

and needs to be investigated in future studies. It would provide an excellent explanation for the exploitation of *U. dioica* as a new host in less favorable habitats.

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Appendix

Table 7 Hierarchical *F*-statistics over all microsatellite loci. (a) German *H. obsoletus* populations from *C. arvensis* and *U. dioica*, grouped according to host-plant affiliation (hierarchical analysis no. 2). (b) European *H. obsoletus* populations from *C. arvensis* and *U. dioica* excluding German populations, grouped according to host-plant affiliation (hierarchical analysis no. 4). For both hierarchical level: F_{ST} = differentiation among all samples, F_{SH} = mean differentiation within host-plant populations caused by geographic structure, F_{HT} = differentiation among host-plant populations. Significant F_{HT} values indicate restricted gene flow among *H. obsoletus* populations infesting *C. arvensis* and *U. dioica*.

(a)

Locus	F_{ST}	F_{SH}	F_{HT}
B82	0.065***	0.026***	0.040*
F56	0.041***	0.021***	0.020*
F84	0.083***	0.029***	0.056*
H120	0.132***	0.022**	0.113**
E96	0.054***	0.020*	0.034*
G85	0.074***	0.031*	0.044**
C147	0.133***	0.067**	0.071**
All loci	0.083***	0.030***	0.054**

(b)

Locus	F_{ST}	F_{SH}	F_{HT}
B82	0.031***	0.037***	-0.006
F56	0.110***	0.115***	-0.006
F84	0.050***	0.056***	-0.006
H120	0.057***	0.067***	-0.010
E96	0.058***	0.047***	0.011
G85	0.084***	0.069***	0.016
C147	0.072***	0.079***	-0.009
All loci	0.064***	0.066***	-0.002

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 8 STRUCTURE analysis of all available populations and percentage of membership for each population assuming $K = 6$ (hierarchical analysis no. 6).

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
D-B09-C	6.8	53.9	11.8	7.4	15.1	4.9
D-L05-C	7.5	61.9	9.2	9.3	10.1	0.2
D-N09-C	22.4	27.4	36.1	0.7	5.2	1.9
D-O07-C	16.6	60.8	0.3	1.9	0.7	10.6
D-P08-C	7.7	68.6	10.9	0.7	4.3	1.5
D-B09-U	81.6	9.6	4.1	1.7	2.1	0.9
D-L06-U	70.7	9.2	7.4	9.2	1.6	1.7
D-N09-U	45.7	1.3	9.4	22.8	5.3	3.7
D-O07-U	69.8	10.4	4.6	5.7	6.8	2.7
D-P06-U	89.8	1.7	3.5	2.7	0.1	1.2
D-R06-U	91.3	3.5	2.4	1.1	1.1	0.5
D-U06-U	81.7	4.6	0.7	2.7	1.7	2.4
F-C09-C	3.1	48.1	15.5	10.4	1.9	3.8
F-H09-L	0.7	0.1	0.1	0.8	1.2	95.3
CH-L10-C	6.4	13.3	74.1	1.9	1.5	2.8
CH-L10-U	5.1	2.5	78.9	11.3	1.4	0.8
CH-A08-U	48.3	6.6	35.3	2.8	5.7	1.3
CH-M09-U	1.4	31.9	27.4	3.4	14.1	9.2
CH-R08-U	10.2	33.2	14.4	12.6	21.1	8.5
I-E07-C	3.6	2.6	23.3	65.4	0.4	1.1
I-C06-C	2.5	7.3	2.4	75.1	9.7	2.9
I-E07-U	1.9	2.6	2.6	81.5	10.1	1.3
I-C06-U	0.3	15.8	13.7	15.7	49.9	1.9
I-R06-U	5.4	0.2	14.8	74.0	2.9	0.9
I-M09-U	4.3	3.3	11.7	64.5	12.5	3.7
I-G09-C	3.1	6.3	18.9	44.7	24.9	2.1
SLO-J04-U	1.9	0.2	11.1	76.9	7.1	0.1
SLO-K04-C	2.8	0.4	5.6	52.5	3.4	1.1
SLO-N06-C	3.6	9.5	13.8	44.3	26.9	1.7
SLO-N06-U	8.1	5.5	12.5	60.0	12.9	0.9
ROM-F08-S	8.4	19.7	12.6	8.1	50.1	1.1
ROM-R09-S	9.8	9.5	7.2	8.4	61.9	3.2
RUS-M09-S	2.8	8.1	9.4	5.2	71.3	3.2
IL-G06-V	1.4	1.5	1.4	1.2	93.3	1.3

Table 9 Pairwise F_{ST} estimates among 34 populations of *H. obsoletus* used in level 6 analysis. Population abbreviations refer to site names in Table 4.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 D-B09-C	0															
2 D-B09-U	0.098	0														
3 D-L05-C	0.016	0.074	0													
4 D-L06-U	0.087	0.010	0.071	0												
5 D-O07-C	0.027	0.081	0.032	0.069	0											
6 D-O07-U	0.057	0.039	0.072	0.011	0.062	0										
7 D-P08-C	0.017	0.089	-0.001	0.082	0.042	0.076	0									
8 D-P06-U	0.114	0.038	0.122	0.014	0.131	0.011	0.126	0								
9 D-N09-C	0.052	0.051	0.044	0.045	0.077	0.063	0.050	0.079	0							
10 D-N09-U	0.051	0.058	0.075	0.035	0.086	0.044	0.070	0.058	0.019	0						
11 D-U06-U	0.103	0.005	0.093	0.011	0.103	0.044	0.105	0.024	0.047	0.051	0					
12 D-R06-U	0.115	0.020	0.080	0.047	0.127	0.053	0.105	0.048	0.051	0.089	0.030	0				
13 CH-A08-U	0.102	0.067	0.102	0.056	0.147	0.079	0.111	0.104	0.039	0.052	0.083	0.114	0			
14 CH-R08-U	0.037	0.098	0.048	0.066	0.049	0.074	0.051	0.100	0.057	0.065	0.092	0.116	0.109	0		
15 CH-M09-U	0.046	0.070	0.041	0.069	0.088	0.077	0.049	0.100	0.048	0.078	0.075	0.100	0.064	0.057	0	
16 CH-LN10-C	0.056	0.098	0.058	0.082	0.087	0.098	0.063	0.122	0.041	0.070	0.091	0.125	0.086	0.055	0.048	0
17 CH-LN10-U	0.070	0.107	0.076	0.096	0.119	0.109	0.078	0.124	0.057	0.080	0.088	0.123	0.101	0.056	0.060	0.010
18 F-H09-L	0.156	0.261	0.201	0.218	0.191	0.235	0.203	0.267	0.209	0.199	0.242	0.324	0.261	0.165	0.186	0.152
19 F-C09-C	0.014	0.080	-0.000	0.077	0.033	0.061	0.007	0.118	0.037	0.070	0.092	0.087	0.077	0.045	0.030	0.047
20 SLO-N06-C	0.063	0.132	0.057	0.104	0.102	0.100	0.063	0.134	0.073	0.093	0.131	0.120	0.108	0.072	0.087	0.066
21 SLO-N06-U	0.094	0.131	0.100	0.091	0.121	0.100	0.088	0.124	0.072	0.065	0.127	0.138	0.127	0.079	0.113	0.088
22 SLO-K04-C	0.044	0.124	0.074	0.084	0.102	0.088	0.066	0.121	0.056	0.052	0.103	0.154	0.120	0.042	0.074	0.052
23 SLO-J04-U	0.109	0.158	0.115	0.110	0.134	0.143	0.101	0.183	0.092	0.091	0.155	0.191	0.149	0.098	0.112	0.094
24 I-G09-C	0.058	0.138	0.067	0.105	0.101	0.115	0.074	0.146	0.090	0.114	0.116	0.156	0.137	0.046	0.086	0.080
25 I-M09-U	0.078	0.110	0.086	0.074	0.095	0.097	0.078	0.114	0.076	0.078	0.092	0.134	0.125	0.035	0.082	0.080
26 I-R06-U	0.110	0.139	0.114	0.112	0.154	0.158	0.109	0.170	0.084	0.096	0.123	0.175	0.133	0.088	0.106	0.078
27 I-C06-C	0.068	0.134	0.089	0.083	0.101	0.070	0.073	0.116	0.072	0.052	0.119	0.137	0.133	0.061	0.089	0.081
28 I-C06-U	0.046	0.099	0.028	0.079	0.057	0.088	0.030	0.129	0.035	0.062	0.093	0.097	0.097	0.038	0.056	0.054
29 I-E07-C	0.116	0.111	0.114	0.100	0.126	0.103	0.111	0.132	0.107	0.121	0.104	0.149	0.128	0.078	0.107	0.127
30 I-E07-U	0.075	0.138	0.092	0.105	0.115	0.097	0.084	0.141	0.074	0.063	0.139	0.150	0.117	0.070	0.102	0.084
31 ROM-F08-S	0.036	0.105	0.052	0.090	0.086	0.074	0.060	0.108	0.045	0.067	0.101	0.093	0.091	0.058	0.059	0.066
32 ROM-R09-S	0.048	0.096	0.052	0.081	0.081	0.092	0.064	0.109	0.067	0.082	0.082	0.114	0.104	0.032	0.051	0.067
33 RUS-M09-S	0.054	0.128	0.065	0.108	0.114	0.096	0.074	0.128	0.076	0.109	0.123	0.142	0.104	0.054	0.046	0.065
34 IL-G06-V	0.076	0.151	0.089	0.130	0.111	0.118	0.097	0.154	0.104	0.102	0.150	0.159	0.133	0.055	0.087	0.100

Population	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
17 CH-LN10-U	0																	
18 F-H09-L	0.205	0																
19 F-C09-C	0.062	0.178	0															
20 SLO-N06-C	0.061	0.202	0.041	0														
21 SLO-N06-U	0.076	0.231	0.077	0.041	0													
22 SLO-K04-C	0.034	0.207	0.045	0.018	0.001	0												
23 SLO-J04-U	0.072	0.243	0.083	0.045	0.014	-0.001	0											
24 I-G09-C	0.049	0.234	0.049	0.057	0.080	0.010	0.071	0										
25 I-M09-U	0.051	0.214	0.059	0.054	0.032	0.006	0.032	0.018	0									
26 I-R06-U	0.038	0.245	0.092	0.070	0.043	0.023	0.012	0.042	0.016	0								
27 I-C06-C	0.060	0.211	0.062	0.035	0.004	-0.014	-0.005	0.061	0.018	0.049	0							
28 I-C06-U	0.057	0.194	0.024	0.043	0.047	0.015	0.052	0.025	0.031	0.051	0.030	0						
29 I-E07-C	0.102	0.274	0.082	0.112	0.108	0.076	0.121	0.067	0.033	0.093	0.086	0.085	0					
30 I-E07-U	0.067	0.223	0.062	0.030	0.007	-0.006	0.024	0.081	0.042	0.061	-0.011	0.065	0.083	0				
31 ROM-F08-S	0.064	0.198	0.023	0.030	0.049	0.001	0.074	0.053	0.062	0.082	0.042	0.026	0.106	0.044	0			
32 ROM-R09-S	0.062	0.185	0.048	0.061	0.089	0.038	0.084	0.034	0.051	0.077	0.068	0.048	0.100	0.095	0.023	0		
33 RUS-M09-S	0.067	0.190	0.037	0.053	0.085	0.018	0.107	0.048	0.077	0.104	0.076	0.054	0.114	0.084	0.017	0.020	0	
34 IL-G06-V	0.117	0.166	0.083	0.097	0.112	0.054	0.117	0.098	0.101	0.133	0.094	0.060	0.161	0.116	0.059	0.045	0.051	0

Chapter 3

Bindweed or nettle: Do the genetic host races of *Hyalesthes obsoletus* differ in their preference?

Abstract

The planthopper *Hyalesthes obsoletus* Signoret (Homoptera: Auchenorrhyncha) is the main vector of stolbur phytoplasma, the pathogen of the grapevine yellows disease bois noir. Even though *H. obsoletus* is considered polyphagous, it has evolved two genetically distinct host races on *Convolvulus arvensis* (field bindweed) and *Urtica dioica* (stinging nettle) in Germany. *Urtica dioica* is a common host in other parts of the distribution range, but it has presumably not been used as a host in Germany until about two decades ago. The regionally novel use of this host coincides with an immense increase in the abundance of bois noir-infected grapevine. The aim of this study was to shed light onto the differentiation process between the two host races. What is the role of olfactory cues in the recognition and orientation of *H. obsoletus* in the search for a host plant and do the two German host races display different responses? Using two assays, a dynamic Y-shaped olfactometer setup and a direct-choice setup, I compared the preference of both races for either host plant. Males and females collected from *C. arvensis* showed a strong preference for their ancestral host in both setups. Specimens collected from *U. dioica*, however, strongly preferred *U. dioica* in the direct-choice assay, but did not exhibit a significant preference for either host in the olfactometer assay. This indicates that host race evolution is still in progress. For the *C. arvensis*-associated race, olfactory clues obviously play a major role in host plant recognition, but for the *U. dioica*-associated race, other factors seem to be important. Additionally, we tested whether stolbur infection of the vector influences its host plant preference. Although not statistically significant, *U. dioica*-associated specimens infected with stolbur phytoplasma tended to choose *U. dioica* in the olfactometer assay, contrary to non-infected specimens, which warrants further studies.

Introduction

The study of host preference of insects in combination with genetic data gives valuable insights into the evolution of host specialization and into the epidemiology of vector-transmitted diseases. These studies are also indispensable for the development of biological control strategies based on infochemicals. When a species' specific preference for certain volatiles is known, these volatiles could be used in volatile-baited traps for monitoring or in some rare cases also for mass trapping of disease-transmitting insects (Gross 2012). As a consequence of exact monitoring, the timing of insecticide application could be improved and thus, the use of insecticides reduced (Gross 2012).

Here, we tested the host preferences in populations of the cixiid planthopper *Hyalesthes obsoletus* Signoret (Homoptera: Auchenorrhyncha). *Hyalesthes obsoletus* was first reported in Germany in 1939 (Wagner 1939) but until about two decades ago, the species was considered extremely rare (Sergel 1986). Since the 1990s, the abundance of *H. obsoletus* has increased extraordinarily, and with it the geographic distribution and incidence of the vectored grapevine yellows disease bois noir, caused by phytoplasma of the stolbur 16SrXII-A group. This increasing abundance coincides with the acquisition of a new host plant by the vector (Johannesen *et al.* 2008).

Convolvulus arvensis (field bindweed) is historically the original host plant of *H. obsoletus* in Germany, but has been replaced by *Urtica dioica* (stinging nettle) as the most widespread host (Langer & Maixner 2004). The results presented in chapter 2 indicate that a host shift occurred from *C. arvensis* to *U. dioica*, with subsequent adaptation of the *U. dioica*-associated specimens to their new host, resulting in two genetically distinct host races. Transplant experiments have shown that German *H. obsoletus* survive significantly longer on their original host plant (Albert 2011). Additionally, populations of *H. obsoletus* associated with *U. dioica* and *C. arvensis* in Germany show morphological (Johannesen *et al.* 2008), behavioral (Grube 2010) and phenological differences (Lux *et al.* 2006). However, it is unknown whether the genetic differentiation between the host races is merely maintained by different phenologies and low dispersal abilities, or by active choice, which could be based on olfactory cues. Knowledge about the preference for certain volatiles could be the first step in developing biological control strategies.

Sharon *et al.* (2005) were to our knowledge the first to conduct olfactory experiments with *H. obsoletus*. They tested the relative preference of *H. obsoletus* collected from eight native plants in Israel for *Vitex agnus-castus* (monk's pepper), *C. arvensis*, and *Vitis vinifera*

(grapevine). Israeli *H. obsoletus* preferred *V. agnus-castus* over the alternative plants. In Germany, however, *V. agnus-castus* is not a known host of *H. obsoletus*, and Germany is the only known region with two genetically distinct host races of *H. obsoletus* (chapter 2).

Kessler *et al.* (2011) conducted two-choice experiments with field-captured as well as laboratory-reared Swiss *H. obsoletus*, which had the choice between *C. arvensis* and *U. dioica.* Adult specimens exhibited a significant preference for *U. dioica.* However, all specimens in this experiment had been collected from *U. dioica* and no genetically distinct host races are evident in Switzerland. Riolo *et al.* (2012) tested the attraction of *U. dioica*-associated Italian *H. obsoletus* to *U. dioica*, *C. arvensis*, *Calystegia sepium* (hedge bindweed), *V. agnus-castus* and *V. vinifera* using a Y-shaped olfactometer. Males were attracted to *V. agnus-castus*, whereas females were attracted to *U. dioica*, when these plants were compared with blank. *C. arvensis* seemed to be neither attractive nor repellent for either sex when compared with blank. Different plants were not tested against each other. Since *U. dioica* is the major host in Italy and no host races have been observed, the females' response is not surprising. For German *U. dioica*-associated specimens, we don't necessarily expect such a strong response, since *U. dioica* is a novel host in this region and German *U. dioica*-associated populations are genetically closer related to German *C. arvensis*- than to Italian *U. dioica*-associated populations (chapter 2). The preference of Italian male specimen for *V. agnus-castus* was unexpected and explained by the authors as an indication of genetically based host choice.

The aim of the present study was to reveal the role of olfactory cues in the recognition and orientation of *H. obsoletus* in the search of a host plant and especially, to study whether the two German host races display different responses. Since *C. arvensis* is the original host plant of *H. obsoletus* in Germany, we expect a stronger preference of the *C. arvensis*-associated specimens to their native host and maybe even a repellent effect of the alternative host. *U. dioica*-associated specimens, on the other hand, might not have developed a strong preference for their new or a strong aversion to their former host because the host shift from *C. arvensis* to *U. dioica* supposedly took place rather recently. In the olfactometer assay, single specimens had the choice between the odors of the two main host plants in Germany - *C. arvensis* and *U. dioica* - or between one host plant and pure air. Each specimen was tested for stolbur infection to analyze the influence of stolbur infection on host preference. To reveal whether other factors than olfactory clues play a role in host recognition, groups of 20 specimens of the same sex were kept in cages with potted *C. arvensis* and *U. dioica* and their host choice was recorded. Microsatellite analyses were

conducted to prove that the tested specimens belong to two genetically distinct host races and to detect possible preference-related sub-structuring within each race.

Materials and Methods

Sampling

We collected *H. obsoletus* by sweeping nets through monoculture stands of their host plants, *C. arvensis* and *U. dioica*. Samples for the olfactometer assay were collected on three days in June 2011 (06/07 from *C. arvensis* in Bacharach and Boppard; 06/15 from *C. arvensis* in Bernkastel-Kues and Boppard and from *U. dioica* in Bernkastel-Kues and Kesten; 06/28 from *U. dioica* in Bernkastel-Kues). Samples for the direct-choice assay were collected on two days in June 2012 (06/22 from *C. arvensis* in Boppard and 07/09 from *U. dioica* in Kesten). It was not possible to collect a sufficient number of specimens from a single syntopic site. However, as has been shown in chapter 2, no genetic sub-structuring between populations of the same host race was found in Germany. Thus, specimens from different sample locations should not bias the results.

Olfactometer assay

We kept *H. obsoletus* in screen-covered boxes containing feeding-plants in climate chambers at 18 °C and about 60 % humidity until they were used in trials. About 2 hours before the test, each individual was transferred to a reaction tube and kept at 8 °C without food in order to increase its motivation.

The host plant preference of 639 *H. obsoletus* specimens was determined by olfactometry according to Pizzinat *et al.* (2011). The olfactometer consisted of a Y-shaped glass tube, 2 cm in diameter. The base was 15 cm long, and the two arms of the olfactometer were each 22 cm in length. Each arm was attached to a flow meter and an odor source, which was either a whole potted *C. arvensis* or *U. dioica* plant or pure air. The test-plants were raised from seed and grown in a greenhouse until use. Each plant was placed inside an inert plastic bag and sealed airtight around the stem. Therefore, only the plant odor and no volatiles contained within the soil were collected. In each case, charcoal-filtered air was forced through each arm of the apparatus and humidified with water. The air entered the plastic bag through a tube, absorbed the plant volatiles and left the bag through another tube. It entered the Y-olfactometer with a constant flow of 145 ml/min. A light source was positioned exactly above the middle of the olfactometer; any additional

light sources were excluded. There were three setups: *C. arvensis* vs. *U. dioica*, *C. arvensis* vs. pure air, and *U. dioica* vs. pure air. Specimens were placed individually in the base tube of the Y-olfactometer and allowed to move upwind towards either of the two arms of the olfactometer. Preliminary experiments had shown that testing each specimen individually worked better than testing groups of five to seven individuals, as in Sharon *et al.* (2005). Trial periods were 5 min in length or until the specimen crossed the "finish line", which was 2 cm into each arm. To compensate for any positional bias, the y-shaped glass tube, together with the position of the test plants, was rotated 180° after every ten individuals. In order to avoid contamination by remaining plant volatiles, the glass tube was washed with ethanol and dried after each experiment. The glass tube was also cleaned between testing male and female specimens to avoid any bias due to an influence of the opposite sex. The significance of host preference was tested for each setup with χ^2 tests for equal distribution (GraphPad Software).

Direct-choice test

All collected specimens were first placed in plastic containers without a plant for about two hours in order to increase their motivation to find a food plant, to decrease the influence of the plant from which they were collected, and to create comparable conditions to the olfactometer setup. After this period, 20 individuals of the same sex and host race were placed in each plastic-screen cage (30 x 30 x 30 cm) containing two potted greenhouse-reared test-plants, *C. arvensis* and *U. dioica* (total number of *C. arvensis*-associated and *U. dioica*-associated specimens, $N = 800$). The test plants were of approximately the same size (about 28 cm high) and placed in opposite sites of the cage. A plastic falcon (8 x 2.5 cm) containing the specimens was placed between the two plants and the lid on top of the falcon was opened. Specimens immediately left the falcon. The distribution of the specimens was recorded for every cage three hours after the start of the experiment and three times a day during the next three days. In total, the distribution of the specimens in each cage was determined ten times. After each count, the specimens were collected and placed again in the falcon between the two plants in order to randomize the distribution; therefore, we considered the ten counts of each cage as independent samples. For the analyses, only specimens that were found on either plant were considered, specimens sitting on the cage netting, escaped, dead or not found were not included.

The consistency of the distribution of specimens over time within each cage was tested with the χ^2 test for homogeneity. If the measures were consistent, they were summed up

for each cage and it was tested whether the observed distribution of *H. obsoletus* on *C. arvensis* and *U. dioica* in each cage deviated from a chance distribution. This was tested with a χ^2 test for equal distribution.

Stolbur infection

We tested all specimens that were used in the olfactometer assay for the discrimination between *C. arvensis* and *U. dioica* for a stolbur infection with the stolbur-specific primers STOL11f2 und STOL11r1. The PCR procedure was as described by Daire *et al.* (1997). The specimens used in the direct-choice assay were not analyzed for stolbur infection because specimens could move freely between the two plants and could not be distinguished individually. A test for infection of the remaining individuals after the ten counts would have been biased by fitness because only about 30 % of the individuals survived until the last count.

Genetic diversity

Population genetic estimates were obtained for 113 individuals of the olfactometer assay. When possible, 20 individuals were chosen randomly from each of the following six groups: Individuals from *C. arvenis* that preferred *C. arvensis*/*U. dioica*/pure air and individuals from *U. dioica* that preferred *C. arvensis*/*U. dioica*/pure air. This setup was chosen to test for a correlation between choice and genetic makeup.

Genetic diversity of populations used in the olfactometer assay was estimated as expected heterozygosity (H_E), allele number (A) and allelic richness (A_R) (FSTAT 2.9.3, Goudet, 1995). Deviations from Hardy–Weinberg (*HW*) proportions and linkage disequilibrium between pairs of loci were analyzed for each population with the web-based version of GENEPOP (Raymond & Rousset 1995), using default settings.

Population differentiation

Host plant influence on differentiation among populations and reflection of host choice in genetic differentiation within each group was estimated using Bayesian clustering (STRUCTURE 2.3.3, Pritchard *et al.* 2000). Analyses of molecular variance (AMOVA) using ARLEQUIN 3.5 (Schneider *et al.* 2000) were conducted to test for genetic structure at three hierarchical levels: host-plant affiliation (F_{HT}), differentiation within groups (F_{ST}), and preference in the olfactometer setup within host race (F_{SH}).

For all STRUCTURE analyses, we chose the admixture model and the option of correlated allele frequencies between populations. These are considered the best settings when populations have similar frequencies due to migration or common ancestry, and therefore have low differentiation and subtle population structure (Falush *et al.* 2003). We performed hierarchical analyses of molecular variance (AMOVA, program ARLEQUIN, Schneider *et al.* 2000) to estimate the level of genetic differentiation between host races and groups of individuals that showed different preferences within each host race. This method apportions genetic variation within and among pre-defined groups and allows quantifying how much of the overall genetic variation can be assigned to the different genetic groups, as revealed with STRUCTURE.

Results

Host preference: Olfactometer assay

To reach a sufficient sample size, two or three locations, respectively, had to be sampled for each host race. Since the specimens from different locations did not differ in their preference (χ^2 test for equal distribution, $p > 0.05$), all specimens collected from *C. arvensis* at different locations were pooled, as well as all specimens collected from *U. dioica*.

Both males and females associated with *C. arvensis* showed a significant preference for the odor of their native host when given the choice between the odors of *C. arvensis* and *U. dioica* ($\chi^2 = 20.056$, $df = 1$, $p < 0.0001$, $N = 72$). Of those individuals that made a decision, 34 males, and 21 females (total 76 %) chose *C. arvensis* over *U. dioica* odor (9 males, 8 females, total 24 %; Figure 8b). 24 males and 30 females did not make a decision. When given the choice between either plant odor and pure air, no preference for either plant was observed (Figure 8a, *C. arvensis* vs. pure air: 21 males and 9 females chose *C. arvensis*, 12 males and 8 females chose air, 6 males and 9 females did not make a decision, $\chi^2 = 2.000$, $df = 1$, $p = 0.1573$, $N = 50$; Figure 8c, *U. dioica* vs. pure air: 10 males and 4 females chose *U. dioica*, 6 males and 5 females chose air, 1 male and 3 females did not make a decision, $\chi^2 = 0.360$, $df = 1$, $p = 0.5485$, $N = 25$).

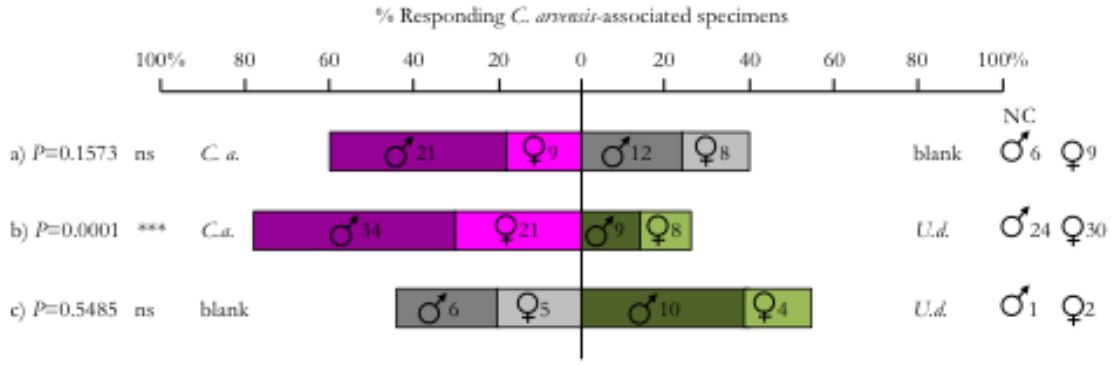

Figure 8 Behavioral responses of *H. obsoletus* collected from *C. arvensis* when given the choice between *C. arvensis* and pure air (a), *C. arvensis* and *U. dioica* (b), and pure air and *U. dioica* (c) in the olfactometer. Percentages of individuals preferring *C. arvensis* are depicted in pink, preferring *U. dioica* in green and pure air in grey. The numbers on the bars indicate the number of males and females that made a choice; the level of significance (males and females combined) is indicated on the left (χ^2 test: ns, non-significant; ***$p < 0.0001$; NC, number of individuals that did not complete a choice.

The specimens collected from *U. dioica* preferred pure air against the odor of *C. arvensis* (Figure 9a, $\chi^2 = 6.119$, $df = 1$, $p = 0.0134$, $N = 59$; 14 males and 6 females chose *C. arvensis*, 23 males and 9 females chose air, 18 males and 43 females did not make a decision). When *U. dioica* specimens were given the choice between pure air and the odor of *U. dioica*, respectively, no preference was detectable (Figure 9c, *U. dioica* vs. pure air: 18 males and 7 females chose *U. dioica*, 23 males and 9 females chose air, 19 males and 44 females did not make a decision). When the odors of both putative host plants were offered simultaneously, females showed a slight, non-significant tendency for *C. arvensis*, whereas male specimens did not show any preference for either host (Figure 9b, *U. dioica* vs. *C. arvensis*: 45 males and 13 females chose *U. dioica*, 47 males and 6 females chose *C. arvensis*, 19 males and 50 females did not make a decision).

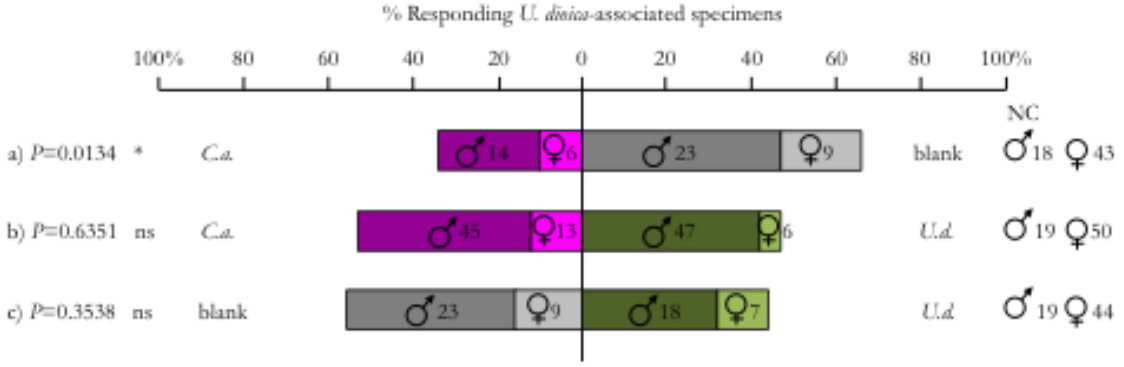

Figure 9 Behavioral responses of *H. obsoletus* collected from *U. dioica* when given the choice between *C. arvensis* and pure air (a), *C. arvensis* and *U. dioica* (b), and pure air and *U. dioica* (c) in the olfactometer. Percentages of individuals preferring *C. arvensis* are depicted in pink, preferring *U. dioica* in green and pure air in grey. The numbers on the bars indicate the number of males and females that made a choice; the level of significance (males and females combined) is indicated on the left (χ^2 test: ns, non-significant; $*p < 0.05$; NC, number of individuals that did not complete a choice.

Twenty-two percent of the specimens from *C. arvensis* and 20 % from *U. dioica* were infected with stolbur. Stolbur infection of *H. obsoletus* did not have a significant effect on host plant preference, but infected *U. dioica*-associated specimens seemed to slightly prefer *U. dioica*, in contrast to uninfected specimen. (*C. arvensis*-associated specimens: $\chi^2 = 0.1370$, $df = 2$, $p = 0.7113$, $N = 72$; *U. dioica*-associated specimens: $\chi^2 = 2.2294$, $df = 2$, $p = 0.1354$, $N = 110$; Figure 10).

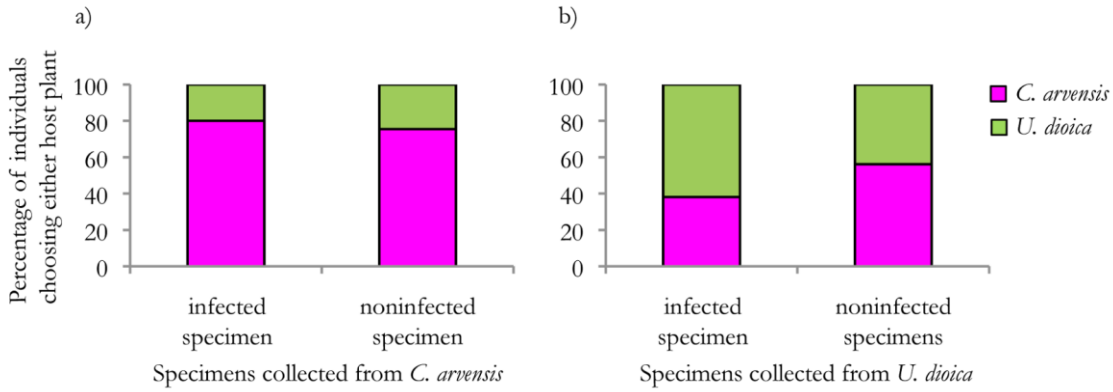

Figure 10 Host plant preference of infected (left bars) and uninfected (right bars) *H. obsoletus* collected from *C. arvensis* (a) and *U. dioica* (b) when given the choice between the odors of *C. arvensis* (pink) and *U. dioica* (green) in olfactometer.

Host preference: Direct-choice assay

For *C. arvensis*- as well as *U. dioica*-associated specimens, no variation in host preference was detected per cage over all ten counts. Therefore the ten counts per cage were summed up and each cage tested separately for equal distribution.

Both male and female *C. arvensis*-associated specimens in all 20 cages significantly preferred their native host *C. arvensis* (χ^2 test for equal distribution, for 18 cages $p < 0.0001$, for two cages $p < 0.05$). In total, of the accumulated counts of males on either plant, 530 individuals were found on *C. arvensis* and 140 individuals on *U. dioica*, the remaining specimens were either undecided, dead, or could not be found. Of the accumulated female counts, 630 individuals were found on *C. arvensis* and 64 individuals on *U. dioica* (Figure 11a).

Urtica dioica-associated specimens in 19 of 20 cages significantly preferred their native host *U. dioica* (χ^2 test for equal distribution, for 16 cages $p < 0.0001$, for three cages $p < 0.01$). In one cage with males, in the accumulated counts 17 individuals preferred *C. arvensis* and 26 preferred *U. dioica*, therefore the trend is the same as in the other cages, but counts were too low to reach significance. For unknown reasons, more individuals died in this cage than in the other cages. In total, of the accumulated counted males, 565 individuals were counted on *U. dioica* and 137 individuals were counted on *C. arvensis*. Of the accumulated female counts, 580 individuals were counted on *U. dioica* and 153 individuals were counted on *C. arvensis* (Figure 11b).

The preference in *C. arvensis*-associated males and *U. dioica*-associated males and females for their respective native host was equally strong (78 - 80 % of those that made a decision; χ^2 test for equal distribution, $\chi^2 = 1.3495$, $df = 2$, $p = 0.5093$). *Convolvulus arvensis*-associated females displayed a stronger preference (91 %) for their native host than *C. arvensis*-associated males and *U. dioica*-associated specimens.

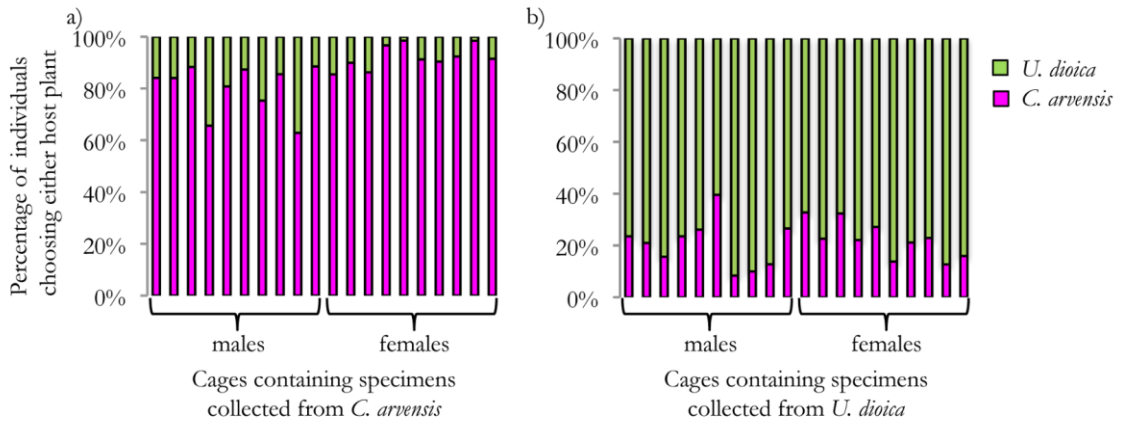

Figure 11 Percentage of individuals collected from *C. arvensis* (a) and *U. dioica* (b) found on *C. arvensis* (pink) and *U. dioica* (green) in each cage (specimens sitting on the cage netting, escaped, dead or not found are not included in the 100 %).

Host preference: Comparison of the two setups

For males in the direct-choice setup and both sexes in the olfactometer setup, the preference of the *C. arvensis*-associated race for the native host was equally strong (72 – 79 % of those that made a decision; χ^2 test for equal distribution, $\chi^2 = 0.7473$, $df = 2$, $p = 0.6882$). Females in the direct-choice test showed an even stronger preference with 91 % choosing *C. arvensis* (Figure 12a).

The *U. dioica*-associated host race differed strongly in response in both setups. In the direct-choice setup, 79 % of the females and 80 % of the males that made a decision chose their native host. In the olfactometer setup, 32 % of the females and 47 % of the males that made a decision chose their native host (χ^2 test for equal distribution, males and females combined, $\chi^2 = 42.6412$, $df = 1$, $p < 0.0001$; Figure 12b).

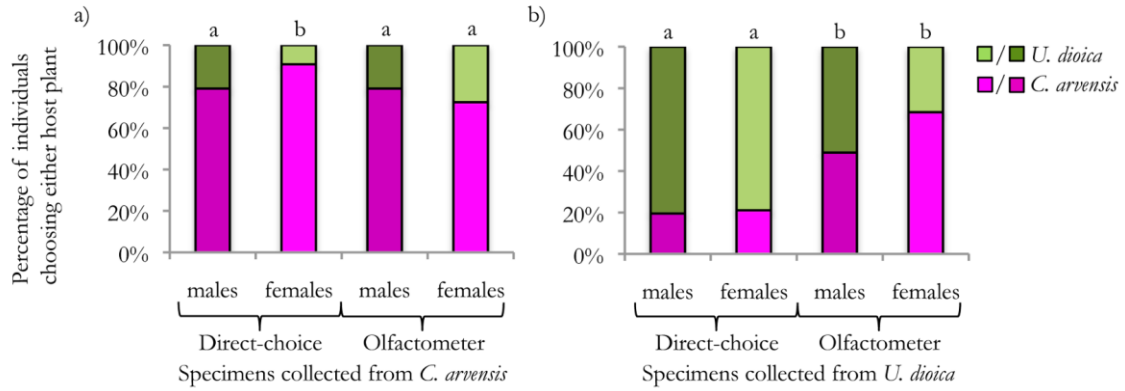

Figure 12 Comparison between the direct-choice setup and olfactometer-setup for both host races. Different letters designate a significantly ($p < 0.05$) different response.

Genetic diversity and HWE

All seven microsatellite loci were polymorphic across all groups, with a mean allelic richness of 7.5. The number of alleles per locus varied from 9 (F56 and H120) to 18 (C147), mean 12. Out of a total of 86 alleles, none were private. Significant linkage disequilibrium was detected for one locus pair in one population (126 pairwise comparisons) after Bonferroni correction (Rice 1989), which is negligible.

Mean expected heterozygosity per population ranged from 0.691 to 0.770 and mean observed heterozygosity from 0.622 to 0.725. Locus C147 was excluded from this analysis, because it was sex-linked (all males were homozygote at this locus). The mean inbreeding coefficient was 0.163. Significant deviations from Hardy Weinberg were recorded in 2 out of 36 possible single locus exact tests after Bonferroni correction ($p < 0.05$). This shows that no locus and no population exhibit an overall deviation from Hardy Weinberg.

STRUCTURE clearly revealed two genetic groups, one consisting of the individuals collected from *C. arvensis* and one consisting of the individuals collected from *U. dioica*. No sub-structuring within groups was detectable. Thus, the host choice within each population in the olfactometer setup was not reflected in genetic differentiation.

AMOVA analysis (ARLEQUIN, Schneider *et al.* 2000) detected a significant genetic structure within the groups at two of the three hierarchical levels considered: host-plant affiliation ($F_{HT} = 0.052$), and differentiation within groups ($F_{ST} = 0.059$). Preference in the olfactometer setup within host race (F_{SH}) was not reflected in genetic differentiation. The differentiation between host-plant associated populations accounted for 88 % of the total variance ($F_{HT}/F_{ST} = 0.881$).

Discussion

The *C. arvensis*-associated *H. obsoletus* showed a very pronounced preference for their native host in both choice-setups (Figure 12a). This has two implications. First, it shows that even though *H. obsoletus* is considered a polyphagous insect using - among many other plants - *C. arvensis* and *U. dioica*, it does show a strong preference for its native plant. This corroborates the finding that many polyphagous insects using a range of hosts throughout their distribution display preference/acceptance hierarchies that vary with climatic or geographical region and even within a population (Hodkinson 1997; Thomas *et al.* 2001). However, not all individuals show the same preference. This explains how a new host race on *U. dioica* could be founded - namely by individuals that differed in their host preference hierarchy from the majority of their population. Second, plant volatiles play a major role in plant detection for *H. obsoletus* - at least for *C. arvensis*-associated specimens. If other factors like visual clues or actual probing of the plants were the decisive factors, we would expect a lower preference in the olfactometer assay than in the direct-choice assay, but the preference was equally strong in both assays. The *U. dioica*-associated *H. obsoletus*, on the other hand, exhibited different reactions in the two setups. The olfactometer assay showed that the evolutionary younger *U. dioica*-associated population has not developed an aversion to its ancestral host's odor. Female specimens even showed a slight but not statistically significant preference for their ancestral host *C. arvensis* (Figure 9b). This effect has been shown for other host race systems as well, e.g. in the butterfly *Euphydryas editha* (Singer 1982) or in the leaf beetle *Chrysomela lapponica* (Gross *et al.* 2004). Singer (1982) proposes that a change in preference is not a prerequisite for a host shift. If the preferred host is scarce, a new population can be founded on a novel host without a change in preference of the adults. In the locations where we collected *H. obsoletus* from *U. dioica*, *U. dioica* was a lot more abundant than *C. arvensis*. Therefore, *C. arvensis* might be higher in the rank order of preferences of *H. obsoletus*, but *U. dioica* probably becomes acceptable if *C. arvensis* is scarce or if intraspecific competition is high. In the direct-choice assay, *H. obsoletus* strongly preferred its native host *U. dioica*. In comparison, this indicates that for *U. dioica*-associated *H. obsoletus*, the tested olfactory clues play a minor role in host acceptance. Since *U. dioica* is most likely a novel host for *H. obsoletus* in Germany, adaptation to it may still be in progress. The results of the olfactory setup, in comparison with the direct-choice test, can be explained in two ways: (1) *Urtica dioica*-associated specimens don't have a clear preference for either tested volatile. The odor of the original host has a repellent effect in comparison to pure air, the odor of the new host seems to be neither attractive nor

repellent. (2) Only part of the population has developed a preference for the novel host and part still prefers the volatiles of the original host, but "changes its mind" after probing the phloem sap. In both cases, additional signals, such as gustatory, visual, or tactile clues, are necessary to cause a preference for one host plant. This also indicates that host discrimination is at least in part genetically based, because if it was predominantly based on learning, olfactory clues should be learned together with visual or gustatory clues. Riolo *et al.* (2012) found that male Italian *H. obsoletus* associated with *U. dioica* actually prefer *Vitex agnus-castus* (monk's pepper) in an olfactometer assay. Like Kessler *et al.* (2011), who tested the preference of Swiss *H. obsoletus* nymphs and adults, Riolo *et al.* (2012) argue that host preference is not only based on associative learning, but also determined by genetic factors. *Vitex agnus-castus* is not found in the Italian agro-ecosystem, but it is the preferred host in Israel (Sharon *et al.* 2005). Since southern and western *H. obsoletus* have a common Levantine origin (Johannesen *et al.* 2008), a genetically determined preference for a host never encountered by the individual before, but used by the ancestral population, seems plausible. It would therefore be interesting to test the reaction of both German *H. obsoletus* host races to *V. agnus-castus*. Since the preference for *U. dioica* was very strong in the direct-choice setup, other discriminating factors like visual and gustatory clues may be of greater importance for host choice in *H. obsoletus* and may therefore be under stronger selection during a host shift and subsequent adaptation. In this case, we expect that the preference for *U. dioica* volatiles will grow stronger with each generation because individuals with the wrong olfactory preference would waste energy by probing the wrong host. The olfactometer assay should be repeated in future years to test this hypothesis.

The genetic analyses proved that the specimens used for the olfactometer tests belonged to two genetically distinct host races, associated with *C. arvensis* and *U. dioica*. It also strengthened the assumption of a genetically based host preference in *H. obsoletus*. However, for a clear distinction between the influence of nature and nurture, multi-generation analyses are necessary.

One point of concern is the relatively low activity of the tested specimens in the olfactometer assay, e.g. 57 % of the *C. arvensis*-associated individuals made a decision when given the choice between the two plants, the remaining specimens did not cross the finish line within the given time. However, an activity below 60 % is not uncommon in olfactory setups with hemipteran insects (e.g. Soroker *et al.* 2004, Mayer *et al.* 2008). This is mainly explained by the artificial setup and handling of the insects, which may cause stress. Another possible reason could be a repellent effect of *U. dioica* odor. Because the

simultaneously offered volatiles of both plants mix at the base of the olfactometer, one of the odors could deter the specimens from entering the base tube at all. In this case, we would expect the same or an even lower motivation in the setup "*U. dioica* versus pure air". But the opposite was true – in this test, 89 % of the specimens made a decision. However, insects react to quantities, qualities, and compositions of certain volatiles. When the odor of two plants is mixed, the composition of volatiles can change in a way that makes the mixture more or less attractive or repellent, respectively. It is therefore possible that the mixture of a repellent and an attractive plant appears more repellent than the repellent plant by itself. The repellent effect of the *C. arvensis* volatile is obvious in the *"C. arvensis* vs. pure air" setup with the *U. dioica*-associated population. Here, the motivation was exceptionally low (49 %) because the repellent volatile mixed with pure air at the base of the olfactometer and deterred the specimens from entering the tube at all. Further studies with single extracted volatiles should reveal which compounds have an attractive or repellent effect on *H. obsoletus*.

The finding that *H. obsoletus* was attracted or repelled by volatile components of several host plants is of major importance for the development of innovative biological control strategies of this important bois noir-vector. By creating insect traps containing attractive components for monitoring flight activity of *H. obsoletus* in vineyards, we can determine the optimal time period for spraying insecticides. Thus, the amount of applied chemical insecticides could be significantly reduced (Gross 2012). Additionally, the combination of attractive and repellent compounds could be used to develop so-called push-and-pull strategies. This biotechnical application consists of dispensers emitting synthetic repellent compounds (push component) and traps supplied with synthetic attractants (pull component). This combination can help to further improve the effectiveness of infochemical-lured traps (Gross 2012). Further analyses are required to identify the chemical compounds that attract or repel *H. obsoletus*.

The second point of concern was the missing individuals in the direct-choice test. Twenty individuals had been placed in each cage, but the number of counted individuals declined over time. Most of the missing individuals likely died and could not be retrieved on the soil in the plant pots. Dead animals on the cage bottom could easily be found, but dead animals on the soil could hardly be spotted and quickly decomposed. Some individuals might also have escaped unnoticed when the cages were opened for the counting. Some live individuals might have been overlooked between the leaves of the

plants, but since each plant was searched very thoroughly, the number should be small and equally distributed between the two host plants.

Stolbur-infection might have a slight influence on the *U. dioica*-associated population; infected males slightly preferred *U. dioica* (10 of 14 individuals that made a choice, 71 %), uninfected males were rather indifferent (41 of 77 individuals that made a choice, 53 %). Unfortunately, sample size was insufficient to reach significance. This aspect deserves further analysis. It has been shown that phytoplasma can convert plants from non-hosts into hosts or turn them into better hosts, but the process by which this happens has not yet been discovered (reviewed by Hogenhout *et al.* 2008). Furthermore, phytoplasma infections can make a host plant more attractive than uninfected ones due to a change in the chemical composition of the emitted volatiles (Mayer *et al.* 2008a, 2008b). If an infection with the *U. dioica*-type stolbur (tuf-type a) actually increases the preference of *H. obsoletus* for *U. dioica*, it would explain the evolution of host races of *H. obsoletus* in Germany. The first switch from *C. arvensis* to *U. dioica* might have happened coincidentally or due to reasons mentioned above, but an infection with stolbur tuf-type a might reduce further gene flow between specimens on *C. arvensis* and *U. dioica* by intensifying their preference for one specific host plant.

Author contributions

Chapter	1	2	3
Original idea	JJ, AS	JJ, AS	MI, JG, MM, JJ
Laboratory work	JL, MI	MI	MI
Data analyses	MI	MI, JJ	MI
Manuscript preparation	MI, JJ, TH	MI, JJ, MM	MI, JG, JJ

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Erklärung

Ich versichere, dass ich meine Dissertation "Host race formation in *Hyalesthes obsoletus* (Signoret 1865)" selbstständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner anderen Quellen und Hilfen als der von mir ausdrücklich bezeichneten bedient habe.

Mühlhausen, im Februar 2013 Miriam Imo