

## ORIGINAL ARTICLE

# Genetic, ecological, behavioral and geographic differentiation of populations in a thistle weevil: implications for speciation and biocontrol

Isabelle Olivieri,<sup>1</sup> Michael C. Singer,<sup>2</sup> Sara Magalhães,<sup>1</sup> Alexandre Courtiol,<sup>1</sup> Yvain Dubois,<sup>1,3</sup> David Carbonell,<sup>1</sup> Fabienne Justy,<sup>1</sup> Patrícia Beldade,<sup>1</sup> Camille Parmesan<sup>2</sup> and Yannis Michalakis<sup>4</sup>

1 Institut des Sciences de l'Évolution, Université Montpellier, Montpellier, France

2 Integrative Biology, University of Texas, Austin, TX, USA

3 CSIRO Biological Control Unit, Campus International de Baillarguet, Montpellier, France

4 GEMI UMR CNRS – IRD 2724, IRD, Montpellier, France

## Keywords

biocontrol, genetic differentiation, host preference, isolation by distance, *Larinus*, local adaptation, specialization, thistles.

## Correspondence

Isabelle Olivieri, Institut des Sciences de l'Évolution, Université Montpellier 2, Place Eugène Bataillon, 34095 Montpellier cedex 05, France. Tel.: 00 33 4 67 14 37 50; fax: 00 33 4 67 14 36 22; e-mail: isabelle.olivieri@univ-montp2.fr

**Present address:** Sara Magalhães, Instituto Gulbenkian de Ciência, Evolutionary Biology Group, Rua da Quinta Grande, 6, 2780-156 Oeiras, Portugal

**Present address:** Patrícia Beldade, Institute of Biology, Leiden University, Kaiserstraat 63, 2311 GP Leiden, The Netherlands

Received: 24 September 2007

Accepted: 5 December 2007

## Introduction

Parasitic species frequently show spatial variation in host use (Fox and Morrow 1981; Thompson 1994), even among habitats with similar arrays of potential hosts (e.g. Singer and Parmesan 1993). The relationship between this variation and parasite genetic differentiation is under increasingly intensive study because of its many spinoffs

## Abstract

Because weevils are used as biocontrol agents against thistles, it is important to document and understand host shifts and the evolution of host-specificity in these insects. Furthermore, such host shifts are of fundamental interest to mechanisms of speciation. The mediterranean weevil *Larinus cynarae* normally parasitizes either one of two thistle genera, *Onopordum* and *Cynara*, being locally monophagous. In Sardinia, however, both host genera are used. We used three types of data to help understand this complex host use: (i) weevil attack rates on the two host genera among 53 different populations in Sardinia and nearby Corsica, (ii) host preference in a lab setting, and (iii) genetic (allozyme) differentiation among weevil populations exploiting the same or different hosts. Using a subset of populations from northern Sardinia, we attempted to relate interpopulation differences in host preference to gene flow among populations by comparing pairwise differences in oviposition preference ( $Q_{st}$ ) and in allozyme frequencies ( $F_{st}$ ). Overall,  $Q_{st}$  and  $F_{st}$  were positively correlated.  $F_{st}$  was positively correlated with geographic distance among pairs of populations using the same host, but not among different-host population pairs. As mating occurs on the hosts, this result suggests reinforcement. Genetic evidence indicates *Cynara* as the ancestral host of the weevils from both islands and our current studies suggest repeated attempts to colonize *Onopordum*, with a successful shift in Corsica and a partial shift in Sardinia. This scenario would explain why in Sardinia the level of attack was higher on *Cynara* than on *Onopordum* and why, when given a choice in the laboratory, Sardinian weevils preferred *Cynara* even when sampled from *Onopordum*. The lability of host shifts in *L. cynarae* supports caution in using these or related weevils as biocontrol agents of exotic thistles.

for the evolution of resource use (Bernays and Chapman 1994; Feder et al. 2003; Ferrari et al. 2006; Xie et al. 2007) and speciation (Feder et al. 1988; Via 1999; McCoy et al. 2001; Dres and Mallet 2002). The speciation aspect has acquired renewed impetus from the recent discovery of several mechanisms that should promote genetic divergence between sympatric populations using different hosts. For example, the preference for a particular host

can be associated with performance on that host (Singer et al. 1988; Hawthorne and Via 2001) and mate choice behavior can be directly driven by host affiliation (Feder et al. 1994; Gotoh and Kubota 1997; Funk 1998; Nosil et al. 2002, 2007). The latter can occur when mate-attraction pheromones are host-derived (Landolt and Phillips 1997; Emelianov et al. 2001) or when males and females show parallel variation in prelighting host choice (Emelianov et al. 2004).

In addition to the knowledge generated by a few model systems (Bush 1994; Via 2001), the study of the processes underlying speciation can be considerably enriched by investigating systems which are on the verge of speciating. For example, comparing the spatial patterns of host preference and of genetic differentiation may provide insight into the transition to speciation. Analyzing factors that determine the host range of an insect is facilitated in species where this host range varies among populations. In this context, the weevil *Larinus cynarae* and the thistles it parasitizes is an excellent system to study the evolution of specialization. Here, we address the question of the geographic scale of specialization and investigate its consequences for genetic differentiation in a system in which speciation has not (yet) occurred.

The issue of host use and genetic differentiation among populations also bears on the choice of potential biocontrol agents. In this context, the use of the weevil–thistle system is particularly relevant, as weevils related to *L. cynarae* have been used as biocontrol agents against thistles (Jordan 1995; Briese 1996; Louda 1998). To choose biocontrol agents wisely, we need to know how insect host ranges evolve, how predictable is the direction of such evolution, and how best to interrogate particular insects about their future evolutionary plans (Strong 1997; Singer 2004; Hufbauer and Roderick 2005; Sheppard et al. 2005). For each group of insects that contains candidates for biocontrol agents, we need to understand the evolution of their host specialization and the mechanics of the host shifts that they undertake. This will assist in predicting the characteristics of parasites, hosts, and their interactions which may make some systems more or less appropriate for biocontrol intervention.

*Larinus cynarae* exhibits strong geographic variation for host use (Briese et al. 1996). In southern France and northern Spain, the weevil feeds exclusively on *Onopordum* species, while it attacks only *Cynara* species in southern Spain, continental Italy (with a few rare exceptions on *Onopordum*, Briese et al. 1996) and Greece. Both host genera are present in each of these regions but only one of them is used, the weevil being thus regionally monophagous (Briese et al. 1996; Y. Michalakis and I. Olivieri, personal observation). Such local

monophagy is well-known in herbivorous insects (Singer 1971; Fox and Morrow 1981; Sheppard et al. 2005). In contrast to this general pattern of regional monophagy, *L. cynarae* attacks species belonging to both genera in Corsica and Sardinia (*Onopordum illyricum* and *Cynara cardunculus*). Both host species flower at the same time and are roughly equally abundant in Sardinia, although relative abundances and phenology of the two genera vary among locations and *Cynara* is essentially absent from the extreme North of the island, as well as from Corsica (I. Olivieri, personal observation).

We report on three kinds of empirical data: (i) weevil attack rates on Corsican and Sardinian populations of both plant genera in the field, (ii) host preferences of experienced and naive insects under experimentally controlled conditions, and (iii) genetic differentiation, assessed by enzyme electrophoresis, among weevil populations exploiting the same or different host species. These different lines of evidence enable us to describe the geographic pattern of host exploitation in the field, to assess the potential of different insect populations to attack one or both host genera, and to investigate how host preference and spatial isolation interact to shape the population genetic structure of this weevil. We discuss the implications of our findings for biological control using this type of organisms.

## Materials and methods

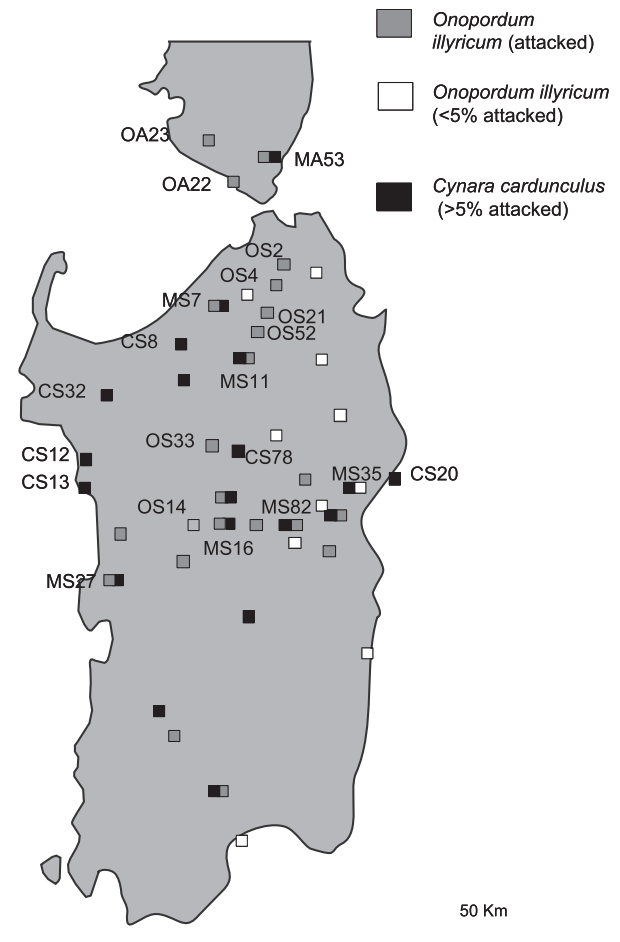
### The natural history of *L. cynarae*

*Larinus cynarae* FAB. (Curculionidae Cleoninae) is a univoltine, sexually reproducing weevil, which feeds, mates, and develops almost exclusively on thistles of the genera *Onopordum* and *Cynara* (Asteraceae: Cardueae) in the circum-mediterranean area. Adult weevils become active in the spring and feed and mate on their host until early summer. During the same period, females lay eggs between the bracts of thistle flower heads. A single egg is laid into each hole that a female has drilled with her snout. In the lab, we observed that females would live for 3–6 weeks, during which they could lay up to 50 eggs (I. Olivieri, personal observation). After hatching, the larvae grow inside the capitula and feed on the developing seeds (Martelli 1948; Michalakis et al. 1993; Briese 1996). Pupation occurs in the capitulum inside a more or less loose cocoon of chewed capitulum tissue. Development from egg to adult lasts about 6 weeks. After completing development, the adults emerge from the dry capitula, often disperse away from the plants, overwinter by undergoing diapause, and appear again the following spring. Adults do not feed from the time of eclosion until breaking diapause in spring and do not survive after the reproductive period.

### Attack rates in the field

When we started this study, we did not know which host(s) would be attacked in the two Mediterranean islands considered here. Both islands are slightly closer to Italy, where *Cynara* is the principal host of *L. cynarae*, than to France, where only *Onopordum* is attacked. The geographic proximity of the two islands suggested that gene flow should frequently occur between them. However, *Cynara* is very rare in Corsica, hence if weevils were present on this island, they would have to parasitize *Onopordum*. Finally, when we discovered that both hosts were attacked in Sardinia and that both could co-occur at the same site, it was initially unclear whether the local monophagy typical for this weevil would be maintained or whether both hosts might actually be used sympatrically. To address these issues, we developed a long-term field study. From 1995 to 2006, weevils were sampled from host plant populations at 40 sites in Sardinia (Fig. 1), including 22 *Onopordum* pure populations (hereafter called 'OS' populations for 'using *Onopordum* in Sardinia'), nine pure *Cynara* populations ('CS' populations for 'using *Cynara* in Sardinia'), and nine mixed stands where both species occurred ('OMS' and 'CMS' populations for 'using *Onopordum* which occurs as a Mixed stand with *Cynara* in Sardinia' and for 'using *Cynara* which occurs as a Mixed stand with *Onopordum* in Sardinia', respectively). At such sympatric sites, plants of both species occurred next to one another. Five additional sites were sampled in Corsica, including four *Onopordum* populations where only *Onopordum* was present ('OA' populations for 'using *Onopordum* in Corsica') and one pair of sympatric plant populations, which represent the only site in Corsica where *Cynara* are present ('OMA' and 'CMA' populations for 'using *Onopordum* which occurs as a Mixed stand with *Cynara* in Corsica' and 'using *Cynara* which occurs as a Mixed stand with *Onopordum* in Corsica', respectively). Because of the unpredictability of host phenology, not all populations were in the right stage for sampling for weevil attacks when visited. Out of 54 populations, 37 were sampled more than once (Table 1). On average each site was visited 2.9 times (SD = 2.1).

In July of each year, a random sample of at least 50 capitula per host species (1–5 capitula per plant) was haphazardly collected from each site sampled that year and brought back to the lab for dissection (in most cases about 100 capitula were dissected). The attack rate on each host was defined as the percentage of capitula that contained at least one weevil: either a well-developed larva (L3, L4, or nymph) or an emerging adult. To compare attack rates in Corsica and Sardinia, to address the effect of the co-occurrence of both plants on host use,



**Figure 1** Map of Sardinia and southern Corsica showing the location of study populations. MS indicates sites where both hosts are used sympatrically in Sardinia; MA indicates a similar site in Corsica. OA and OS indicate pure populations of *Onopordum illyricum* in Corsica and Sardinia. CS indicate pure populations of *Cynara cardunculus*. Identified sites are those studied in the host preference experiments and/or the allozyme study. Sites MS27, MS16 and MA53 were more particularly considered for the effect of host in sympatry, whereas populations OA22, OA23, MS11, CS16, OS27, CS27, and CS32 were used in Experiment 2 to study the effect of diet on host preference. Gray squares indicate attacked populations of *O. illyricum*, white squares, unattacked populations of *O. illyricum*, and black squares attacked populations of *C. cardunculus*.

and to take into account temporal variation, we tested whether attack rates on the two host species were significantly variable among population types (CS, CMS, OS, OMS, OA, OMA, and CMA) and years using a generalized linear mixed-effects model (hereafter called GLMM). *Population type* and *year* were considered as fixed effects. Data from a given population (i.e. weevils sampled at a given site from a given host species) across years are not necessarily independent. To control for this potential lack

**Table 1.** List of studies performed for each population, indicating (i) year of studies for field attack rates (96–99 = 1996–1999; 00–06 = 2000–2006); year of experiment (sample sizes) for (ii) host preference and (iii) allozyme study. For the 1996 host-preference experiments, the sample size corresponds to the number of replicates (single female or pair of females).

Population type	Population	Field attack	Host choice non-naive weevils	Host choice naive weevils diet = original host	Host choice naive weevils diet = alternative host	Enzyme polymorphism
OA	OA22	95, 96, 98, 02, 06	96 (12), 00 (6), 04 (37)	01 (4), 03 (13)	03 (10)	96 (35)
OA	OA23	95, 98, 01, 02, 03, 06	00 (4), 04 (19)	03 (9)	03 (10)	96 (33)
OA	OA1	98, 06	–	–	–	–
OA	OA24	98, 01, 02, 06	–	–	–	–
CMA	CMA53	04, 06	00 (4), 04 (5)	–	–	–
OMA	OMA53	98, 99, 04	04 (7)	–	–	–
CS	CS8	95, 96, 98, 99, 06	96 (7), 00 (7)	–	–	96 (28)
CS	CS9	96	–	–	–	–
CS	CS12	95, 96, 98, 99, 01, 03	96 (10), 00 (4)	02 (2)	–	96 (44)
CS	CS13	95, 06, 98, 01, 02, 03, 06	–	02 (4)	–	–
CS	CS20	95, 06	–	–	–	96 (37)
CS	CS32	98, 01, 02, 03, 04, 06	00 (4)	01 (1), 02 (5), 03 (20)	02 (3), 03 (12)	–
CS	CS48	98	–	–	–	–
CS	CS49	98	–	–	–	–
CS	C78	01	–	02 (5)	–	–
OS	OS2	95, 96, 98, 02, 04	96 (7)	03 (2)	–	96 (30)
OS	OS4	95, 96, 01, 02, 03, 04, 06	96 (34), 00 (7)	01 (3)	01 (4), 02 (2)	96 (39)
OS	OS6	98	–	–	–	–
OS	OS14	95, 96, 98, 01, 02, 04, 06	96 (9), 00 (14)	01 (3), 02 (3), 03 (4)	02 (4)	96 (14)
OS	OS15	96, 98	–	–	–	–
OS	OS21	95, 96, 98, 01, 02	96 (12)	02 (6)	02 (4)	96 (16)
OS	OS28	96	–	–	–	–
OS	OS29	96	–	–	–	–
OS	OS33	98, 01, 02, 06	–	02 (2)	02 (2)	–
OS	OS34	98	–	–	–	–
OS	OS38	98	–	–	–	–
OS	OS42	98	–	–	–	–
OS	OS41	03	–	–	–	–
OS	OS44	98	–	–	–	–
OS	OS45	98	–	–	–	–
OS	OS46	98	–	–	–	–
OS	OS47	98	–	–	–	–
OS	OS52	99	04 (17)	01 (5)	–	–
OS	OS60	01, 02, 03	–	–	–	–
OS	OS79	04	–	–	–	–
OS	OS83	06	–	–	–	–
OS	OS84	03	–	–	–	–
CMS	CMS7	98, 99, 02	–	–	–	–
OMS	OMS7	95, 96, 98, 02, 04, 06	96 (10)	01 (2)	01 (3)	–
CMS	CMS11	98, 99, 01, 02, 03, 06	–	–	–	–
OMS	OMS11	98, 99, 01, 02, 06	–	01 (3), 02 (4), 03 (7)	02 (4), 03 (10)	–
CMS	CMS16	95, 96, 98, 99, 01, 02, 04, 06	96 (8), 00 (11)	02 (8), 03 (16)	02 (6), 03 (10)	96 (30)
OMS	OMS16	95, 96, 98, 01, 02, 06	00 (5)	01 (3)	01 (1)	–
CMS	CMS25	96, 98, 01	–	–	–	–
OMS	OMS25	96, 98	–	–	–	–
CMS	CMS27	96, 98, 03, 04	00 (8)	04 (4)	04 (5)	–
OMS	OMS27	96, 98, 99, 03, 04	–	04 (11)	04 (14)	–
CMS	CMS35	98, 01	–	02 (7)	02 (4)	–
OMS	OMS35	98	–	–	–	–
OMS	OMS40	98, 04	–	–	–	–
CMS	CMS80	04, 06	–	–	–	–
OMS	OMS80	04, 06	–	–	–	–
CMS	CMS82	01, 02, 03	–	02 (3), 03 (11), 04 (1)	–	–
OMS	OMS82	01, 02, 03	–	–	02 (1)	–

of independence, and thus to avoid pseudo-replication, we considered the effect of *population* as a random effect, as suggested by Pinheiro and Bates (2000). The number of attack rates per population type was too small to estimate the interaction term *population type:year*. Models were computed with lme4 Package of R, using the *lmer* function (Bates and Sarkar 2007). We assumed a binomial error associated with logit link function. The significance of the effects was assessed by comparing the described model with and without each fixed effect using chi-squared tests on differences in deviance; all models were fitted using unrestricted maximum likelihood estimation (method = ML) and keeping the same random effects, as suggested by Crawley (2007). Pairwise comparisons between population types were computed using the *pvals.fnc* function from the *Language R* package (Baayen 2007), which performs 10 000 MCMC simulations to estimate *P*-values.

### Host preference experiments

To understand the observed patterns of attack in the field, we performed several host preference experiments, classified into two main types described below.

#### *Experiment 1: host preferences of experienced insects*

In June 1996, six weevil populations on *O. illyricum* (five sardinian populations, OS2, OS4, OMS7, OS14, OS21, and one population from Corsica, OA22), and three on *C. cardunculus* in Sardinia (CS8, CS12, CMS16) were sampled during their oviposition period (see Fig. 1 for the location of these populations). Twenty to 50 adult weevils were collected haphazardly on host plants at each site and brought back alive to the CSIRO laboratory in Montpellier to be subjected to oviposition preference experiments. In the lab, each weevil was fed with the same plant species from which it had been collected. Plant material was collected in southern France. Host preference was tested by introducing one or two females (with one male added per female) into cages in which two to four fresh ramets of *C. cardunculus* and of *O. illyricum* had been transplanted in sand, at about 10–20 cm one from another. Each ramet bore one to three capitula in the early blooming stage and the capitula of each host had approximately the same size. Weevils were left in the cage for 2 days, and the number of eggs on each capitulum was counted at the end of this period. Overall, seven to 34 replicates were performed for each population (mean = 12.3, SD = 8.4, see Table 1 for sample size per population). The total number of females tested was 208 (109 experiments with 1 or 2 females tested) and the total number of eggs was 593. Thus, each female laid about three eggs in 2 days, close to what

would be observed in natural conditions (Martelli 1948). The preference of each weevil (or pair of weevils combined) is expressed as the ratio of the number of eggs laid on *Onopordum* over the total number of eggs laid in the cage.

In 2000 and 2004, the same experiment was performed with females sampled in June from various populations (in 2000: OA22, OA23, OS4, OS14, CS8, CS12, CMS16, OMS16, CS32, CMS27, CMA53 in 2004: OS52, OA22, OA23, OMA53, and CMA53, see Table 1 for sample sizes). After feeding on leaves from its original plant species, each female was transferred individually to plastic containers in which she was offered a simultaneous choice between the two hosts. Each container had a single capitulum of each host species of approximately the same size. Capitula were replaced every 2 days. The old capitula were removed and the eggs on them counted.

From 2001 to 2003, as well as in 2004 for one site, the same experiment was performed, but with adult weevils that had been gathered as pupae in the previous year. As seems to occur in natural conditions, the insects did not feed prior to diapause. They were kept at 4°C till April. Diapause was broken by placing the weevils at room temperature and providing them with the host plant on which they had been sampled. Fifteen populations were studied this way with a total of four to 72 weevils per population (OA22, OA23, OS14, OS21, OS52, OMS11, OMS16, OMS27, CS13, CS32, CS78, CMS16, CMS27, CMS35, and CMS82) (Fig. 1, see Table 1 for sample size). For six other populations (CS12, OS2, OS4, OS33, OMS7, and CMS11), sample size was lower than four, but their inclusion or exclusion from the analysis did not affect the results. We analyzed the above dataset in several ways described below.

#### *Variation for host preference*

Using the above dataset, we tested the hypothesis that host preference was independent of population type (CS, CMS, OS, OMS, CMA, OMA, or OA), using a GLMM as previously described with *population type* as a fixed effect and *year* and *population* as random effects. To study the interaction between *year* and *population type* in a meaningful way, we would need a more balanced study. We assumed a binomial error weighted by the total number of eggs laid by each female or each replicate (pair of females).

We also tested the hypothesis that host preference was independent of the host species on which weevils had been collected, using a GLMM (see above) with *host* as a fixed effect and *year* and *population* as random ones. Because there was a single (sympatric) population of *Cynara* in Corsica, and because host preference was found to vary between the two islands (in particular between

populations using *Onopordum*), we performed this last comparison within Sardinia only.

To compare the divergence among populations for host preference with that for allozymes (see below), we defined a 'preference distance' between two populations ( $Q_{st}$ ) as a phenotypic analog of the standardized variance of gene frequencies ( $F_{st}$ ): if  $p_i$  is the observed mean proportion of eggs laid per female or per replicate on *Onopordum* for population  $i$  in the preference experiments,  $Q_{st}$  between any two populations  $i$  and  $j$  is calculated as  $\frac{\text{Var}(p)}{[p(1-p)]}$ , with  $\text{Var}(p)$  the observed variance of  $p$  among the two populations,  $p$  the arithmetic mean of  $p_i$  and  $p_j$  and  $p(1-p)$  the maximum value of  $\text{Var}(p)$ . We calculated  $Q_{st}$  among pairwise populations for the 1996 dataset, so as to compare these preference distances with geographic distances, as well as with genetic distances obtained in the allozyme study described later ( $F_{st}$ ). As Wright (1969) has shown, when the variance of a given selectively neutral quantitative trait is determined by many additive gene effects, the genetic differentiation among populations generated by genetic drift will be equivalent to that at the underlying genes (QTL), or at any neutral locus. This theoretical background has been used to identify traits undergoing homogeneous or heterogeneous selection, for which the amount of genetic differentiation would be smaller or larger, respectively, compared to that observed for likely neutral loci (Bonnin et al. 1996; McKay and Latta 2002; Le Corre and Kremer 2003). Further, a positive correlation between  $Q_{st}$  and  $F_{st}$  can be interpreted as evidence either for a genetic basis of the quantitative trait, or for a covariation between  $F_{st}$  and some environmental factor which also affects  $Q_{st}$ .

#### *Sympatric sites: association between host plant and preference*

Because we found differences between populations of weevils sampled from different hosts, we also tested for preference differences between weevils that could use different hosts in their field site. We used weevils from three sites where both hosts occurred and were attacked (Sympatric sites: MS16 and MS27 in Sardinia, and MA53 in Corsica, see Table 1). We tested for the effects of *host*, *population*, and their interaction on host preference using a generalized linear model (GLM). We used *glm* function of R with a quasibinomial family as error structure and an  $F$ -test to check for the effect of *host*, as suggested by Crawley (2007, p. 578). Using a quasibinomial family allows the model to estimate a dispersion parameter which will scale the nominal variance to take into account departure from a true binomial error (McCullagh and Nelder 1989, p. 124–128). To study the effect of *host* within each population, GLMs were subsequently computed for each population.

#### *Experiment 2: test for induction of host preference in naive insects*

In 2002, 2003, and 2004, we estimated the effects of diet on oviposition preferences of individuals from seven populations, with the aim of understanding the causes of the preference variation among populations revealed in Experiment 1. We used naive adult weevils that had been collected as pupae in the previous year. After diapause was broken, half the weevils from each test population were fed with *Cynara* and the other half with *Onopordum*. After they had fed and mated for about 2 weeks on their test diet, the females were transferred individually to plastic containers in which each female was offered the choice between the two hosts as in the previous experiment. There were two Corsican populations from *Onopordum* (OA22 and OA23), two Sardinian populations from *Onopordum* occurring at a sympatric site (OMS11 and OMS27) and three Sardinian populations from *Cynara* (CMS16, CMS27, and CS32), two of which were sympatric with *Onopordum*. Population OMS11 was sampled in both 2001 and 2002, and tested the following years. For this population, data across years were pooled. Populations OMS27 and CMS27 were sampled in 2003 and studied in 2004. The other populations were sampled in 2002 and studied in 2003. At least 10 females were tested per diet, apart for population CMS27 where only five females were tested on *Onopordum* and four on *Cynara* (see Table 1 for sample sizes).

Because we had studied few populations for each host, we did not study the *host:diet* interaction. Instead we tested for the effects of *population*, *diet*, and their interaction on host preference by a GLMM as previously described for the first experiment. Here, *population*, *diet*, and their interaction are the fixed effects, and *host* is considered as a random effect to control for potential confounding effect of differences among hosts. To study the effect of *diet* within each population, GLM were subsequently computed for each population, with an  $F$ -test to test for the effect of *diet*.

#### **Enzyme polymorphism**

In July 1995, several hundred mature capitula were sampled from eight Sardinian populations (OS2, OS4, OS14, OS21 on *Onopordum*, and CS8, CS12, CMS16, CS20 on *Cynara*, see Fig. 1) known to have been attacked the previous year, as well as two Corsican populations (populations OA22 and OA23), and brought back to the laboratory in Montpellier. Emerging insects were killed in liquid nitrogen and stored frozen at  $-80^{\circ}\text{C}$  until being processed for enzyme polymorphism using the methods previously described for *Larinus* (Michalakis et al. 1992; Briese et al. 1996). Overall, 272 weevils were scored for 10

polymorphic loci, of which seven were highly polymorphic at the level of the species, and five at the level of Sardinia (see Appendix).

Differentiation over all samples and within each host were tested using Fisher's method for combining probability tests. Unbiased estimates of the associated  $P$ -values were calculated using the Markov chain method computed by GENEPOP version 3.4 (Raymond and Rousset 1995). Wright's  $F_{st}$ -statistics ( $F_{st}$ ) (Wright 1951) were estimated by the estimator  $\hat{\theta}$  of Weir and Cockerham (1984). We also used the GDA software (Lewis and Zaykin 2001) to perform a hierarchical ANOVA, to compare the amount of variation within and among hosts in Sardinia. Confidence intervals for  $\theta_S$  (among populations within hosts), and  $\theta_P$  (among host species) were obtained by bootstrapping over loci (Lewis and Zaykin 2001).

The correlation between  $F_{st}$  and pairwise differences in host preference between populations ( $Q_{st}$ ) also studied in Experiment 1 (all eight populations studied for allozymes but population CS20), or of any of these two pairwise distance matrices and geographic distance were tested with Mantel's test (Mantel 1967) using Pearson's correlation coefficient as the test statistic. To test the significance of the correlation between  $F_{st}$  or  $Q_{st}$  and geographic distance depending on whether pairs of populations used the same or different host plant, we used a randomization test by modifying the standard Mantel's test procedure to account for the particular structure of the distance matrices being handled. For populations using different host plants, each of the two distance matrices (one for geographic distances, the other for  $F_{st}$  or  $Q_{st}$ ) are rectangular (with populations on *Onopordum* in, e.g. columns and populations on *Cynara* in rows), and we randomly combined rows and columns of one of them (1000 permutations each time). In the case of populations on the same host plant, for each distance there were two symmetric matrices (relative to the diagonal), each of them corresponding to one host plant. We independently combined the rows and columns of both matrices for one of the distances and then combined the randomly generated matrices to calculate Pearson's coefficient. The two-sided  $P$ -value of the test is calculated as the proportion of sampled permutations where the absolute value of the correlation coefficient is greater than or equal to the observed absolute value.

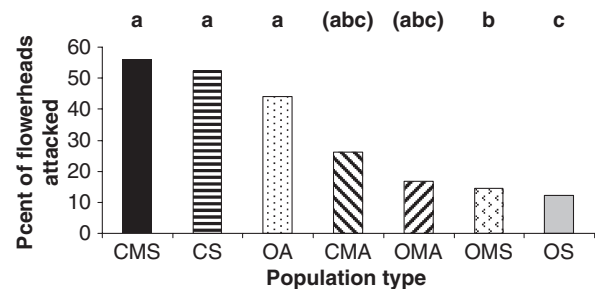
To interpret our results and determine the ancestral host of the Sardinian and Corsican weevils, we used enzyme data from Briese et al. (1996) on weevils from Spain, southern France, Italy and Greece, and a subset of our own data (seven loci out of 10, corresponding to the first seven in Appendix), to reconstruct a distance tree at the scale of the mediterranean basin. The

species *Larinus latus*, specialized on *Onopordum* (assumed to be the ancestral host of *L. cynarae* by Briese et al. 1996), was used as outgroup. We used the PHYLIP 3.57 package (Felsenstein 1994). The program SEQBOOT was used to produce 1000 datasets by bootstrapping over loci; GENDIST was used to compute the Cavalli-Sforza distance, and for each dataset the tree was constructed using the neighbor-joining method. The program Consense allowed the reconstruction of the consensus tree.

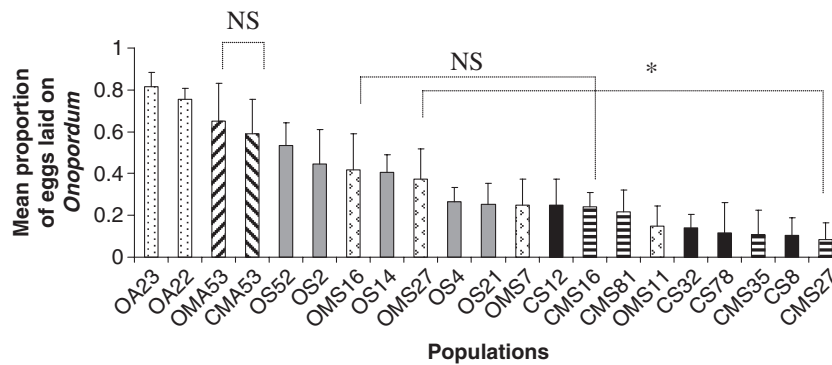
## Results

### Attack rates in the field

The attack rate (proportion of capitula with at least one larva having reached the third instar) varied widely among years, plant species and plant populations, ranging from 0% to 100%. On average, 33% of capitula were attacked in plant populations that were used as hosts (sample size above 50, usually 100). The mean attack rate in 2001 (43.5%) was particularly high, and that in the record heat-wave year of 2003 particularly low (15.9%). As a result, the effect of year was highly significant ( $\chi^2 = 456.90$ , 8 d.f.,  $P < 0.0001$ ). We also found a significant effect of population type ( $\chi^2 = 41.05$ , 6 d.f.,  $P < 0.0001$ ), with Sardinian populations of *C. cardunculus* and pure Corsican populations of *O. illyricum* significantly more heavily attacked than Sardinian populations of *O. illyricum* (Fig. 2, shared letters among population types indicate nonsignificant differences; all significant differences had  $P < 0.007$ ).



**Figure 2** Mean attack rates (percent of capitula containing at least one larva or emerging adult) per population type : CMS and CS: *Cynara cardunculus* from, respectively, sympatric and single-species sites in Sardinia; OA: pure *Onopordum illyricum* from Corsica; CMA and OMA: *C. cardunculus* and *O. illyricum* from the unique sympatric site in Corsica; OMS and OS: *O. illyricum* from, respectively, sympatric and single species sites in Sardinia. Each bar shows the average attack rate over 1–7 years of data. Letters over each bar indicate significant differences among population types: shared letters indicate a lack of significant difference (see text). Letters over CMA and OMA are only indicative, as these types are represented by a unique population.



**Figure 3** Spatial variation. Pattern of host preference in all experiments performed between 1996 and 2004 with weevils fed with the host plant from which they were sampled. Each bar represents the mean proportion of eggs laid per female per population on *Onopordum*, relative to the number of eggs laid on either *Onopordum* or *Cynara*. Values <0.5 indicate a preference for *Cynara*. Error bars (SD) are given under the assumption of a Binomial distribution of the number of eggs laid on each host per each female. Bars connected by dotted lines indicate sympatric weevil populations on each host. Differences among these bars were tested separately (Stars indicate a significant the effect of the original host NS = nonsignificant differences). Original host: Sardinian *Onopordum illyricum* occurring in pure (OS) or mixed stands (OMS); Corsican *O. illyricum* in pure stands (OA); Sardinian *Cynara cardunculus* in pure (CS) or mixed stands (CMS); *O. illyricum* and *C. cardunculus* at the unique sympatric site in Corsica (OMA and CMA).

## Host preference experiments

### Variation of host preference (Experiment 1)

Figure 3 shows the pattern of host preference over all experiments performed between 1996 and 2004 with weevils fed with the host plant from which they were sampled. The effect of population type was significant ( $\chi^2 = 31.3$ , 6 d.f.,  $P < 0.0001$ ). In Corsica (dotted and hatched bars), the mean proportion of eggs laid on *Onopordum* varied from 59% (CMA53) to 81% (OA23). Thus, overall, weevils preferred *Onopordum* in Corsica. Conversely, in Sardinia, the mean proportion of eggs laid on *Onopordum* varied from 8% for a naturally-*Cynara* feeding weevil population at a sympatric site (CMS27) to 53% for populations which naturally fed on *Onopordum* (OS52). Thus, regardless of their original host and location, Sardinian weevils generally preferred to oviposit on *Cynara*, or showed no preference ( $z$ -test,  $z = -9.4$ ,  $P < 0.0001$ ). However, within Sardinia, there was a significant difference in preferences between weevils from the two host plant origins, with populations naturally found using *Cynara* more strongly preferring *Cynara* compared to populations naturally found on *Onopordum* (with average proportion of eggs laid on *Onopordum* of, respectively 14% and 34%;  $\chi^2 = 8.97$ , 1 d.f.,  $P = 0.0027$ ).

### Sympatric sites: association between host plant and preference (Experiment 1)

We specifically tested the effect of host at three sympatric sites (indicated Fig. 3 by horizontal lines linking populations). As weevils from these three sites had significantly different preferences ( $F_{2,87} = 10.78$ ,  $P < 0.001$ ), we tested

for the effect of *Host* within each site. At sites MA53 and MS16 there was no trend for a difference in preference between weevils sampled from the two hosts (MA53:  $F_{1,14} = 0.004$ ,  $P = 0.95$ ; MS16:  $F_{1,49} = 0.027$ ,  $P = 0.87$ ), and at site MS27 there was a large and significant trend for weevils from one host genus to prefer that same genus in experimental preference trials ( $F_{1,21} = 5.12$ ,  $P = 0.034$ ).

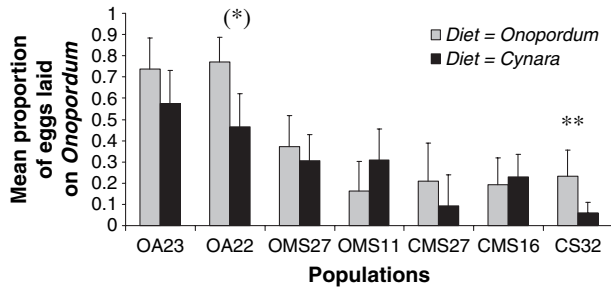
### Test for induction of host preference in naive insects (Experiment 2)

There was no significant main effect of the weevils diet on their oviposition patterns ( $\chi^2 = 0.64$ , 1 d.f.,  $P = 0.43$ ) across all populations (Fig. 4). However, five out of seven populations showed the same trend of increasing preference towards the host they had previously experienced as a diet. Furthermore, there was a significant interaction of population and experimentally-controlled diet ( $\chi^2 = 33.31$ , 6 d.f.,  $P < 0.0001$ ). Host preference of weevils during oviposition trials was strongly and significantly influenced by previous experimentally-manipulated diet in only one population (CS32) ( $F_{1,30} = 6.55$ ,  $P = 0.016$ ). In a second population (OA22), there was a weaker and nonsignificant tendency for induction of preference ( $F_{1,21} = 2.48$ ,  $P = 0.13$ ), whereas experimental diet did not significantly influence host preference by weevils from other populations ( $F < 0.69$ ,  $P > 0.42$ ) (Fig. 4).

### Relationship of preference distance to geographic distance in Experiment 1

Over the eight populations studied for host preference in 1996, no significant relationship between preference distance and geographic distance was found (permutation



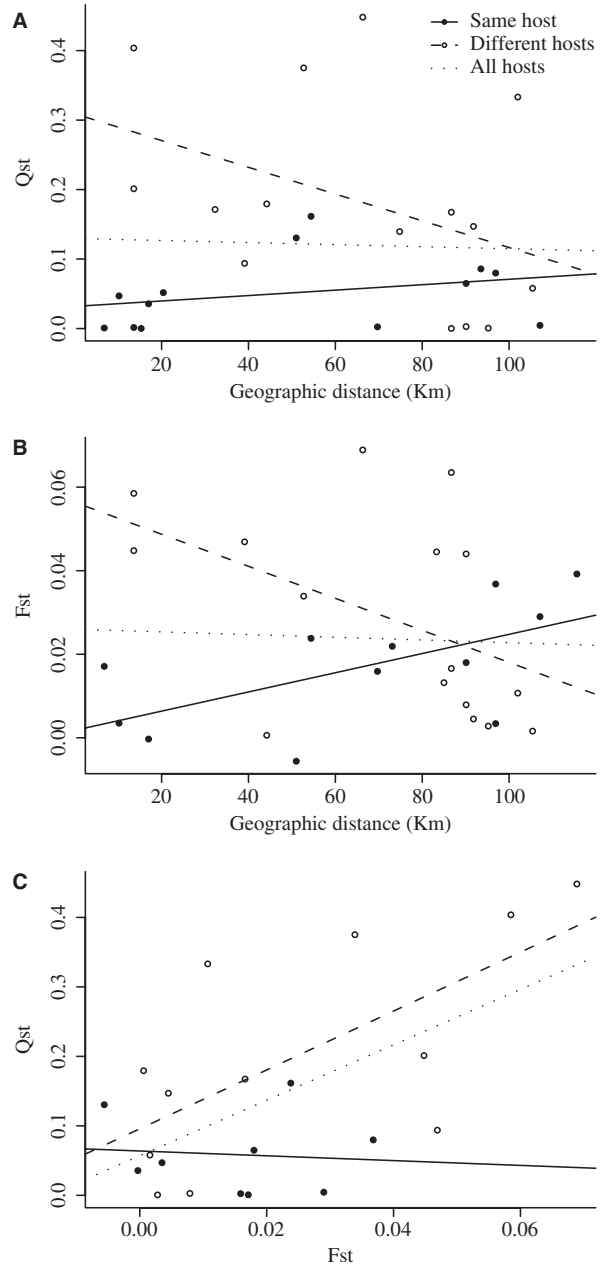


**Figure 4** Effect of host population and diet on host preference. The bars indicate the proportion of eggs laid on *Onopordum illyricum* in host preference experiments. Weevils were sampled in the field in July 2002, overwintered in a cold chamber, fed with either host (black bars: *Cynara*; gray bars: *Onopordum*) in May 2003 and tested in June 2003. Sympatric sardinian populations OMS27 and CMS27 were sampled in 2003 and tested in 2004. Population OMS11 was sampled in both 2001 and 2002, and tested in 2002 and 2003. Error bars (SD) are given under the assumption of a Binomial distribution of the number of eggs laid on each host. Original host: Corsican *O. illyricum* (OA23 and OA22); Sardinian *O. illyricum* (OMS11, OMS27); Sardinian *Cynara cardunculus* (CMS27, CMS16, CS32). The number of females tested was above 10 per diet for populations OA23, OA22, OMS11, OMS27 and CMS16. It was between 4 on *Cynara* and 5 on *Onopordum* for population CMS27. Stars above a population indicate a significant effect of diet on its mean host preference. \*\* $P = 0.016$ ; \* $P = 0.13$ .

test,  $r = -0.03$ ,  $P = 0.89$ ) (Fig. 5A). The sign of the correlation was positive (but still nonsignificant) when only those pairs of populations collected on the same host species were considered ( $r = 0.28$ , permutation test,  $P = 0.54$ ), and negative when we considered only those pairs of populations in which each member of the pair used a different host species ( $r = -0.44$ , permutation test,  $P = 0.26$ ) (Fig. 5A). This last trend was essentially because of weevils at two sites (population OS14 on *Onopordum* and populations CS8 on *Cynara*), which showed unusually strong preferences for the hosts that they used (Fig. 3).

**Enzyme polymorphism**

There was a weak though significant differentiation among populations of the two islands considered together ( $F_{st} = 0.040$ , Fisher probability test,  $P < 0.001$ ), as well as among Sardinian populations ( $F_{st} = 0.022$ , Fisher probability test,  $P < 0.001$ ). The average  $F_{st}$  among pairs of populations was larger between populations on different hosts than between populations exploiting the same host (mean  $F_{st} = 0.029$  and  $0.017$ , respectively). However, a hierarchical ANOVA (GDA) suggested that among-host differentiation was not significantly different from 0 ( $\theta_p = 0.006$ , CI obtained by bootstrapping over loci:  $-0.001-0.014$ ) whereas within-host differentiation was significantly positive ( $\theta_s = 0.025$ , CI:  $0.004-0.050$ ).



**Figure 5** Relationship between various measures of divergence between pairs of populations within Sardinia. Gray symbols: pairs of populations exploiting the same host plant species. Dark symbols: pairs of populations exploiting different host plant species. Regression lines are purely indicative as most of the correlations are not significantly different from 0. (A) Host preference ( $Q_{st}$ ) and geographic distances. Overall,  $r = -0.03$ , one-tailed  $P = 0.89$ ; within same host-plant species:  $r = 0.28$ ,  $P = 0.54$ ; among host-plant species:  $r = -0.44$ ,  $P = 0.26$ . (B) Genetic differentiation ( $F_{st}$ ) and geographic distances. Overall,  $r = -0.03$ ,  $P = 0.91$ ; within same host plant species:  $r = +0.61$ ,  $P < 0.0001$ ; among host-plant species:  $r = -0.49$ ,  $P = 0.14$ . (C) Host preference ( $Q_{st}$ ) and genetic differentiation ( $F_{st}$ ). Overall,  $r = +0.59$ ,  $P = 0.02$ ; within same host plant species:  $r = -0.08$ ,  $P = 0.74$ ; among host-plant species:  $r = +0.67$ ,  $P = 0.05$ .

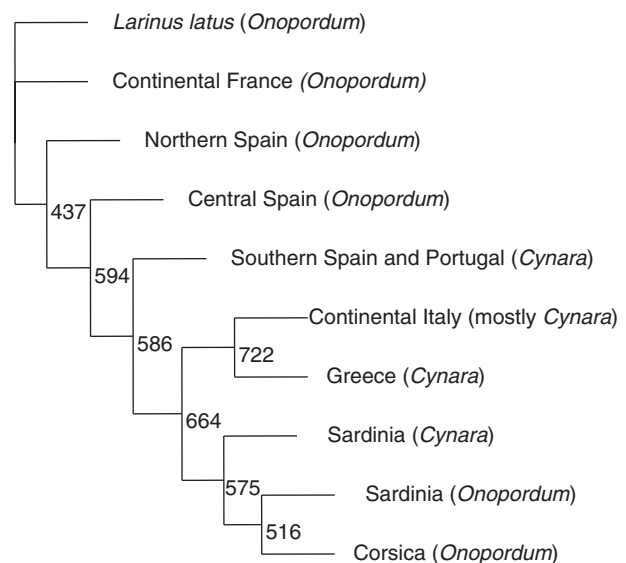
Within Sardinia, there was no significant correlation between genetic distance and geographic distance (Fig. 5B,  $r = -0.03$ , Mantel test,  $P = 0.91$ ). However,  $F_{st}$  and geographic distance between sites became positively and significantly correlated when only those pairs of populations exploiting the same host were analyzed (Fig. 5B,  $r = 0.61$ , permutation test,  $P < 0.0001$ ). On the other hand, when we considered only those pairs of populations collected on different host species, we found a (nonsignificant) negative correlation between  $F_{st}$  and geographical distance (Fig. 5B;  $r = -0.49$ , permutation test,  $P = 0.14$ ), a pattern similar to the relationship between  $Q_{st}$  and geographic distance (Fig. 5A). Thus, for population pairs exploiting different host plants in Sardinia, the genetic differentiation between geographically closely located populations was just as high as that between populations separated by large distances.

Although the overall differentiation was small in Sardinia, a significant positive correlation was observed between preference distance  $Q_{st}$  and  $F_{st}$  (Fig. 5C,  $r = 0.59$ , Mantel test,  $P = 0.02$ ), suggesting that host preference has affected the genetic structure of the weevil metapopulation, or vice versa. This relationship was even stronger when we analyzed only those pairs of populations sampled on different hosts ( $r = 0.67$ , permutation test,  $P = 0.05$ ), whereas it was no longer significant when only same-host population pairs were considered ( $r = -0.08$ , permutation test,  $P = 0.74$ ).

We used allozyme polymorphism to study the likely origin of Sardinian and Corsican populations. We used data from the present work and previously-published data on a subset of the same loci (Briese et al. 1996). We found that both Sardinian and Corsican populations were more closely related to populations specialized on *Cynara* sp. (western Italy and southern Spain), than to populations specialized on *Onopordum* sp. (N. Spain and S. France) (Fig. 6). This phylogeographic pattern gives an explanation for our finding that Sardinian weevils exhibited a general tendency to prefer *C. cardunculus*, regardless of the host they naturally used.

## Discussion

We begin our discussion by drawing together our accumulated evidence from patterns of allozyme and preference variation to infer the current processes involved in generating spatial and temporal patterns of attack by our study species on its two host genera. Subsequently, we discuss the implications of our findings for gene flow and host-range evolution. We then ask how results such as ours may contribute to making informed decisions about the potential risks posed by exotic insects used in biocontrol programs.



**Figure 6** Distance tree based on Cavalli-Sforza genetic distances among Mediterranean populations of *Larinus cynarae*, based on enzyme polymorphism (seven loci). Bootstrap values are indicated at each node. The related species *Larinus latus*, which only feeds on *Onopordum* sp., is used as outgroup.

## Preferences of Sardinian weevils

In contrast to the regional monophagy exhibited over most of its range, *L. cynarae* weevils exploit both host plant genera in Sardinia. However, *Cynara* plants were generally more heavily attacked than *Onopordum* (Fig. 2). The preference experiments under controlled conditions corroborated field observations. Overall, Sardinian weevils preferred *Cynara* to *Onopordum*. However, when given the choice, weevils from populations that used *Onopordum* in the field laid more eggs on this species than weevils from populations using exclusively *Cynara*. These experimental results show that behavioral differences exist among populations.

## Positive correlation between genetic distance and preference distance

The present study is the first to show a quantitative relationship between a continuously varying host preference and a continuously varying genetic divergence. The relationship we found was a positive correlation (Fig. 5C). One can ask what are the mechanisms underlying this positive correlation between  $Q_{st}$  and  $F_{st}$ . First, marker polymorphism could be directly involved in host preference. Indeed, there is some evidence that allozyme polymorphism might not always be neutral with respect, e.g. to assortative mating (Feder and Filchak 1999). However, the same correlation pattern was observed when we used

microsatellite data (F. Justy and I. Olivieri, unpublished data). Therefore it is likely that the observed pattern does not reflect that of particular genes under selection. Another possibility is that genetic differentiation is a direct consequence of host preference: weevils from populations which exhibit different preference may be less likely to encounter each other than insects from populations with similar preference. In this case, gene flow among populations with different host preference would be more restricted compared to that among populations with similar preference. Alternatively, it could be argued that host preference, just as allozyme variation, is neutral and behaves just like any neutral marker (Jimenez-Ambriz et al. 2007, and references therein for examples of *Fst*–*Qst* studies). However, as differentiation at allozyme loci is much lower than differentiation for host preference, preference is most likely under diverging selection. Another possibility would be that host preference is not genetically determined and that *Qst* simply reflects phenotypic plasticity. However, in this case, the strong correlation between *Qst* and *Fst* would remain unexplained.

Although environmental influences on preference, such as the induction demonstrated here, are frequent in beetles, genetic influences typically exist alongside them, leading to significant heritability of oviposition preference (Tucic and Seslija 2007; and references therein) and rapid response to artificial selection (Fricke and Arnqvist 2007).

Other authors have shown how host preference might mediate genetic divergence between host-races (Rice 1985; Duffy 1996; Craig et al. 1997; Feder et al. 1997; Ferrari et al. 2006; Frantz et al. 2006). Indeed, assortative mating based on host preference is expected to lead to genetic differentiation (Feder et al. 1988, 1997; McPheron et al. 1988; Craig et al. 1993). Since *L. cynarae* do mate on their host plant, this mechanism is likely.

#### Host preference and genetic differentiation: a role for reinforcement

Assuming that *Fst* reflects current gene flow, our results suggest that, among populations on different hosts, gene flow among nearby sites is at least as low as that among distant sites, whereas among same-host populations isolation by distance occurs. Indeed, although the overall differentiation among populations is small, there is a tendency for pairs of populations using different hosts to be more genetically distinct than pairs using the same host. More importantly, the two types of population pairs show strikingly different patterns of association between *Fst* and geographic distance. In the Sardinian dataset, the significant positive correlation between *Fst* and geographic distance, expected under the standard isolation-by-distance scenario, is observed among same-host

population pairs. However, this correlation disappears or even becomes negative when we consider only population pairs using different hosts (Fig. 5B).

The trend toward a negative correlation between *Fst* or *Qst* with geographic distance among different-host populations suggests that these populations actually exchange fewer genes than populations further apart. One possible explanation for this pattern is that increased host fidelity has been directly selected for in areas of sympatry or parapatry, as a premating barrier to lessen cross-breeding between weevils associated with *Onopordum* and *Cynara*. Thus, the pattern could correspond to a process of reproductive reinforcement (Butlin 1987; Noor 1999) to reduce the production of less fit hybrids between populations specialized on alternative host plants. Note, however, that we have no evidence yet for hybrids having a low fitness.

The results from our host preference experiments suggest that (i) learning affects host preference differently across populations, and (ii) reinforcement does not systematically occur in sympatric populations (Fig. 3). This variation may be caused by the patterns of variation of the populations themselves in the field. Indeed, thistle or weevil populations are not stable entities. Throughout the 10 years of sampling, some populations have disappeared and/or they have been (re)colonized, suggesting that local extinctions or bottlenecks of plant and/or weevil populations are frequent (I. Olivieri, personal observation). When a population becomes either very scarce or temporarily extinct, it may be recolonized by immigrants from the same host or from the alternate host. When colonization occurs from the alternate host, this may blur the effect of reinforcement. However, we expect a bias towards same-host colonizations as occurs in other oligophagous insects (Hanski and Singer 2001).

Overall, the pattern of host preference appears as one of small isolated populations displaying a mosaic of levels of attack, with repeated attempts to colonize a novel host (*Onopordum*), seemingly leading to selection for reproductive isolation, as suggested by the unexpected patterns of local genetic differentiation. It will be very interesting to follow the evolution of these populations, some of which might prove to be a natural example of speciation mediated by reinforcement on host preference.

#### Phylogeographic scenario and ongoing adaptation on alternative hosts

Over most of its range *L. cynarae* is monophagous on either *Onopordum* or *Cynara*, even when both hosts are available. This monophagy is brought about by strong host preferences: in experimental trials French females laid 94% of their eggs on *Onopordum* and females from southern Spain specialized on *Cynara* laid 95% of their

eggs on plants of this genus (Y.D. and I.O., unpublished data). In an open-field experiment, females from Greece specialized on *C. cardunculus* did not lay any eggs on *Onopordum* (Briese et al. 1995).

The existing evidence suggests that Sardinia was colonized by *Cynara*-exploiting weevils. The higher field attack rates on *Cynara* compared to *Onopordum* (Fig. 2), in conjunction with the distance tree based on enzyme polymorphism (Fig. 6) indicate that these weevils were primarily adapted to *Cynara*. Further, most insects collected on *Onopordum* laid more eggs on *Cynara* than on *Onopordum* when given the choice (Fig. 3). This also supports the scenario of an ongoing host-shift from *Cynara* to *Onopordum*, as other studies have also found a host shift to be followed by a lingering preference for the traditional host remaining among insects using the novel host (Singer et al. 1993; Berlocher and Feder 2002).

If *L. cynarae* are indeed undergoing a host-shift from *Cynara* to *Onopordum*, they are returning to the host identified as the ancestral host of their taxonomic group (according to Briese et al. 1996). This would not be surprising. Janz and Nylin (1998) showed that, in butterflies, a higher tendency to recolonize ancestral hosts helps to explain the apparent large-scale conservatism in the patterns of association between insects and their host plants, patterns which at the same time are flexible on a more detailed level. There are several other examples of such evolutionary conservatism (Thompson 1993; Futuyma et al. 1994; Futuyma and Mitter 1996; Fox et al. 1997).

Our results confirm that the members of the Curculionid taxon Cleoninae can indeed undergo multiple colonizations and radiations on the Cynaroideae, as previously suggested by Zwölfer and Herbst (1988). Geographic variation of insect diet implies its rapid evolution (Singer 1971; Funk and Bernays 2001). Altogether, our current results confirm the great flexibility and evolutionary potential of host preference in these weevils, as has been shown in other insects (e.g. see Taber 1994; Feder et al. 1997; and Messina 2004).

### Implications for biological control

Thistles are important weeds, rendering thistle–weevils potentially important biocontrol agents. Within thistles (tribe Cynarae) there are 16 species of economic importance as noxious weeds in several temperate countries (Schroder, 1980, cited in Petney 1988). *Onopordum* is an introduced pest in Australia, and subject to biological control by *Larinus latus*, a species closely related to *L. cynarae* (Michalakis et al. 1992). Two other seed-head weevils, *Larinus minutus* and *L. obtusus*, have been released in North America in the 1990s to control *Centaurea diffusa* (Groppe et al. 1990; Groppe 1992; Jordan

1995; Lang et al. 1996) and *Centaurea 'maculosa'* (or rather *C. stoebe*, Ochsmann 1999). *Larinus curtus* has been introduced in California in 1992 as an agent against *Centaurea solstitialis* (Turner et al. 1988; Groppe et al. 1990; Sobhian and Fornasari 1994) (see <http://cecalaveras.ucdavis.edu/starthistle.htm>).

One of the most notorious examples of ill-advised biological control involves yet another thistle-head weevil, *Rhinocyllus conicus*, that was introduced against slender thistles (*Carduus pycnocephalus* and *C. tenuiflorus*) from 1968 onwards in the United States and Canada, and that was later found attacking rare, endemic species of the native American flora (Louda et al. 1997, 2005; Strong 1997; Louda 1998; Russell and Louda 2005, Russell et al. 2007).

The history of *R. conicus* shows the importance of understanding the ecological and evolutionary causes and consequences of host-specificity and host shifts prior to making artificial introductions. Despite this cautionary tale, biological control research is continuing unabated. When control is successful its economic impact can be enormous, as in the recent dramatic success of an introduced weevil in clearing water hyacinth from Lake Victoria (Wilson et al. 2007).

Evidently, one should be more cautious when using insects for biocontrol than were the enthusiasts who introduced *R. conicus*, which was already known to have a fairly wide host range (Strong 1997). To assess the risk to native species posed by biocontrol agents, we need to be able to predict their likely evolutionary trajectories. How can this be approached? Recent reviews by Hufbauer and Roderick (2005) and Sheppard et al. (2005) express considerable optimism that the problems are now well-enough understood that if current knowledge were applied uniformly, attack on nontarget plants could be effectively avoided. For example, these authors note that regulations now require introductions to be made from a specific population that has been tested for its potential host range, not just from a species from which some populations have been tested.

There are still, however, some very basic questions to which we do not have answers, such as: 'is there a lower risk when a sample is taken from a strictly monophagous species than from a strictly monophagous population of a species with geographic variation of diet?' (Singer 2004). Although it might seem intuitively obvious that insects in taxonomic groups with strictly monophagous species are less likely to indulge in host shifts, this might still not be true. In groups of strictly monophagous species, each host shift must have been associated with a speciation event. This is true regardless of the direction of cause and effect, i.e., whether the host shifts trigger the speciation events or whether the speciation events facilitate the host shifts.

But this does not necessarily mean that host shifts are rarer in groups with strict monophagy. It could be, on the contrary, that these groups have higher rates of speciation but the same rate of host-shifting as groups containing regionally-monophagous species. This is a testable hypothesis (Singer 2004).

Even if we knew whether we should restrict the search for biocontrol agents to totally monophagous species or also include regional monophagy, the present study illustrates the practical difficulty of classifying species as strictly or regionally monophagous. If *L. cynarae* were studied superficially, it would probably be recorded as completely monophagous. If the study were extended broadly enough geographically, the weevil would be recorded as using two hosts, but always locally monophagous. It is only with luck and extensive work that one finds there are spots in its distribution where its diet is fluctuating, flexible, and probably rapidly-evolving. How many strictly monophagous species are there, and how many that are recorded as monophagous would turn out not to be so with sufficient study? In any case, it seems that weevils contain both species that are strictly monophagous and those that are regionally so, as in the present case.

In the case of *L. cynarae*, the more detailed the investigation undertaken, the broader and more flexible the diet appears to be. However, there are cases where the exact opposite occurs and detailed molecular investigation reveals a supposed generalist insect species as a cluster of cryptic species with narrow diets. Hebert et al. (2004) titled their DNA-barcoding study of neotropical skippers 'Ten species in one' while Fumanal et al. (2004a,b) discovered that an apparently generalist European weevil actually comprised two morphologically identical species, a generalist and a specialist. When this occurs, previously unsuspected candidate biocontrol agents can be revealed and made available for study. Overall, recent work including that reported here, suggests that even in insect groups regarded as suitable for biological control, the factors that influence host range may not yet be well-enough understood to give us the necessary confidence to predict future evolution of introduced agents. Nonetheless, we consider that pursuit of the ability to make these predictions remains a worthwhile enterprise.

## Acknowledgments

Logistic support for early host preference experiments was provided by CSIRO Biological Control Unit of Montpellier. M. Volovitch, J. Mangeant, A. Dobson, A. Olivieri, M.-C. Quillet, I. Bonnin, B. Colas, and P. Belladj provided practical as well as moral support in the field. D. Briese and C. Espiau provided the original dataset

on enzyme polymorphism for mainland populations. M. Marquine, provided technical help for enzyme polymorphism study. Discussions with J. Shykoff, A. Sheppard, D. Briese, C. Espiau, and T. Thomann were very helpful. Benoît Facon helped edit a latest version of the manuscript. M. Kirkpatrick helped a lot in clarifying the results and the ideas presented in this paper. Comments from two anonymous reviewers were very helpful. Financial support was provided by the Ministère de l'Environnement. SM was funded by a Marie Curie European Grant. YM acknowledges support from CNRS and IRD. This is publication ISEM-2007-141 of the Institut des Sciences de l'Evolution de Montpellier.

## Literature cited

- Baayen, R. H. 2008. *Analyzing Linguistic Data: A Practical Introduction to Statistics*. Cambridge University Press, New York.
- Bates, D., and D. Sarkar. 2007. *Lme4: Linear Mixed-Effects Models Using S4 Classes*. URL <http://cran.r-project.org>. R package version 0.99875-2.
- Berlacher, S. H., and J. L. Feder. 2002. Sympatric speciation in phytophagous insects: moving beyond controversy? *Annual Review of Entomology* **47**:773–815.
- Bernays, E. A., and R. F. Chapman. 1994. *Host-Plant Selection by Phytophagous Insects*. Chapman & Hall, Inc., New York, London, 312 pp.
- Bonnin, I., J.-M. Prosperi, and I. Olivieri. 1996. Comparative spatial structure of markers and quantitative characters in a selfing plant species, *Medicago trunculata* (Leguminosae). *Genetics* **143**:1795–1805.
- Briese, D. T. 1996. Life history of the *Onopordum capitulum* weevil *Larinus latus* (Coleoptera: Curculionidae). *Oecologia* **105**:454–463.
- Briese, D. T., A. W. Sheppard, and J. M. Reifenberg. 1995. Open-field-host-specificity testing for potential biological control agents of *Onopordum* thistles. *Biological Control* **5**:158–166.
- Briese, D. T., C. Espiau, and A. Pouchot-Lermans. 1996. Micro-evolution in the weevil genus *Larinus*: the formation of host biotypes and speciation. *Molecular Ecology* **5**:531–545.
- Bush, G. L. 1994. Sympatric speciation in animals: new wine in old bottles. *Trends in Ecology and Evolution* **9**:285–288.
- Butlin, R. 1987. A new approach to sympatric speciation. *Trends in Ecology and Evolution* **2**:310–311.
- Craig, T. P., J. K. Itami, W. G. Abrahamson, and J. D. Horner. 1993. Behavioral evidence for host-race formation in *Eurostigma solidaginis*. *Evolution* **47**:1696–1710.
- Craig, T. P., J. D. Homer, and J. K. Itami. 1997. Hybridization studies on the host races of *Eurostigma solidaginis*: implications for sympatric speciation. *Evolution* **51**:1552–1560.
- Crawley, M. J. 2007. *The R Book*. John Wiley, New York.

- Dres, M., and J. Mallet. 2002. Host races in plant-feeding insects and their importance in sympatric speciation. *Philosophical Transactions of the Royal Society of London Series B – Biological Sciences* **357**:471–492.
- Duffy, J. E. 1996. Species boundaries, specialization, and the radiation of sponge-dwelling alpheid shrimp. *Biological of the Linnean Society* **58**:307–324.
- Emelianov, I., M. Dres, W. Baltensweiler, and J. Mallet. 2001. Host-induced assortative mating in host races of the larch budmoth. *Evolution* **55**:2002–2010.
- Emelianov, I., F. Marec, and J. Mallet. 2004. Genomic evidence for divergence with gene flow in host races of the larch budmoth. *Proceedings of the Royal Society of London Series B – Biological Sciences* **271**:97–105.
- Feder, J. L., and K. E. Filchak. 1999. It's about time: the evidence for host plant-mediated selection in the apple maggot fly, *Rhagoletis pomonella*, and its implications for fitness trade-offs in phytophagous insects. *Entomologia Experimentalis Et Applicata* **91**:211–225.
- Feder, J. L., C. A. Chilcote, and G. L. Bush. 1988. Genetic differentiation between sympatric host races of the apple maggot fly *Rhagoletis pomonella*. *Nature* **336**:61–64.
- Feder, J. L., S. B. Opp, B. Wlazlo, K. Reynolds, W. Go, and S. Spisak. 1994. Host fidelity is an effective pre-mating barrier between sympatric races of the apple maggot fly. *Proceeding of the National Academy of Sciences U S A* **91**:7990–7994.
- Feder, J. L., J. B. Roethele, B. Wlazlo, and S. H. Berlocher. 1997. Selective maintenance of allozyme differences among sympatric host races of the apple maggot fly. *Proceedings of the National Academy of Sciences U S A* **94**:11417–11421.
- Feder, J. L., S. H. Berlocher, J. B. Roethele, H. Dambroski, J. J. Smith, W. L. Perry, V. Gavrilovic *et al.* 2003. Allopatric genetic origins for sympatric host-plant shifts and race formation in *Rhagoletis*. *Proceeding of the National Academy of Sciences U S A* **100**:10314–10319.
- Felsenstein, J. 1994. *PHYLIP (Phylogeny Inference Package)*. Department of Genetics, University of Washington, Seattle, WA.
- Ferrari, J., H. C. J. Godfray, A. S. Faulconbridge, K. Prior, and S. Via. 2006. Population differentiation and genetic variation in host preference among pea aphids from eight host plant genera. *Evolution* **60**:1574–1584.
- Fox, L. R., and P. A. Morrow. 1981. Specialization: species property or local phenomenon? *Science* **211**:887–893.
- Fox, C. W., R. C. Stillwell, A. R. Amarillo-S, M. E. Czesak, and F. J. Messina. 2004. Genetic architecture of population differences in oviposition behavior of the seed beetle *Callosobruchus maculatus*. *Journal of Evolutionary Biology* **17**:1141–1151.
- Frantz, A., M. Plantegenest, L. Mieuzyet, and J. C. Simon. 2006. Ecological specialization correlates with genotypic differentiation in sympatric host-populations of the pea aphid. *Journal of Evolutionary Biology* **19**:392–401.
- Fricke, C., and G. Arnqvist. 2007. Rapid adaptation to a novel host in a seed beetle (*Callosobruchus maculatus*): the role of sexual selection. *Evolution* **61**:440–454.
- Fumanal, B., J.-F. Martin, R. Sobhian, A. Blanchet, and M.-C. Bon. 2004a. Host range of *Ceutorhynchus assimilis* (Coleoptera: Curculionidae), a candidate for biological control of *Lepidium draba* (Barssicaceae) in the USA. *Biological Control* **30**:598–607.
- Fumanal, B., J. F. Martin, and M. C. Bon. 2005. High throughput characterization of insect morphocryptic entities by a non-invasive method using direct-PCR of fecal DNA. *Journal of Biotechnology* **119**:15–19.
- Funk, D. J. 1998. Isolating a role for natural selection in speciation: host adaptation and sexual isolation in *Neochlamisus bebbianae* leaf beetles. *Evolution* **198**:1744–1759.
- Funk, D. J., and E. A. Bernays. 2001. Geographic variation in host specificity reveals host range evolution in *Uroleucon ambrosiae* aphids. *Ecology* **82**:726–739.
- Futuyma, D. J., and C. Mitter. 1996. Insect–plant interactions: the evolution of component communities. *Philosophical Transactions Royal Society of London B* **351**:1361–1366.
- Futuyma, D. J., J. S. Walsh, T. Morton, D. J. Funk, and M. C. Keese. 1994. Genetic variation in a phylogenetic context: responses of two specialized leaf beetles (Coleoptera: Chrysomelidae) to host plants of their congeners. *Journal of Evolutionary Biology* **7**:127–146.
- Gotoh, T., and M. Kubota. 1997. Population dynamics of the citrus red mite, *Panonychus citri* (McGregor) (Acari: Tetranychidae) in Japanese pear orchards. *Experimental and Applied Acarology* **21**:343–356.
- Groppe, K. 1992. *Larinus obtusus* Gyll. (Col.: Curculionidae): A Candidate for Biological Control of Diffuse and Spotted Knapweed. International Institute of Biology control, Délé-mont, Switzerland, 46 pp.
- Groppe, K., R. Sobhian, and J. Kashefi. 1990. A field experiment to determine host specificity of *Larinus curtus* Hochhut (Coleoptera: Curculionidae) and *Urophora sirunaseva* Hg (Diptera, Tephritidae), candidates for biological control of *Centaurea solstitialis* L. (Asteraceae) and *Larinus minutus* Gyllenhal, a candidate for biological control of *Centaurea maculosa* Lam. and *Centaurea diffusa* Lam. *Journal of Applied Entomology* **110**:300–306.
- Hanski, I., and M. C. Singer. 2001. Extinction-colonization dynamics and host plant choice in butterfly metapopulations. *American Naturalist* **158**:341–353.
- Hawthorne, D. J., and S. Via. 2001. Genetic linkage of ecological specialization and reproductive isolation in pea aphids. *Nature* **412**:904–907.
- Hebert, P. D. N., E. H. Penton, J. M. Burns, D. H. Janzen, and W. Hallwachs. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptus fulgerator*. *Proceedings of the National Academy of Sciences U S A* **101**:14812–14817.

- Hufbauer, R. A., and G. K. Roderick. 2005. Microevolution in biological control: mechanisms, patterns and processes. *Biological Control* 35:227–239.
- Janz, N., and S. Nylin. 1998. Butterflies and plants: a phylogenetic study. *Evolution* 52:486–502.
- Jimenez-Ambriz, G., C. Petit, I. Bourrie, S. Dubois, I. Olivieri, and O. Ronce. 2007. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* 173:199–215.
- Jordan, K. 1995. Host specificity of *Larinus minutus* Gyll. (Col., Curculionidae), an agent introduced for the biological control of diffuse and spotted knapweed in North America. *Journal of Applied Entomology* 119:689–693.
- Landolt, P. J., and T. W. Phillips. 1997. Host plant influences on sex pheromone behavior of phytophagous insects. *Annual Reviews of Entomology* 42:371–391.
- Lang, R. F., J. M. Story, and G. L. Piper. 1996. Establishment of *Larinus minutus* Gyllenhal (Coleoptera: Curculionidae) for biological control of diffuse and spotted knapweed in the western United States. *Pan-Pacific Entomologist* 72:209–212.
- Le Corre, V., and A. Kremer. 2003. Genetic variability at neutral markers, quantitative trait loci and trait in a subdivided population under selection. *Genetics* 164:1205–1219.
- Lewis, P. O., and D. Zaykin. 2001. *Genetic Data Analysis: Computer Program for the Analysis of Allelic Data. Version 1.0 (D16c)*. Free program distributed by the authors over the internet from <http://lewis.eeb.uconn.edu/lewishome/software.html>.
- Louda, S. M. 1998. Population growth of *Rhinocyllus conicus* (Coleoptera: Curculionidae) on two species of native thistles in prairie. *Environmental Entomology* 27:834–841.
- Louda, S. M., D. Kendall, J. Connor, and D. Simberloff. 1997. Ecological effects of an insect introduced for the biological control of weeds. *Science* 277:1088–1090.
- Louda, S. M., T. A. Rand, A. E. Arnett, A. S. McClay, K. Shea, and A. K. McEachern. 2005. Evaluation-of ecological risk to populations of a threatened plant from an invasive biocontrol insect. *Ecological Applications* 15:234–249.
- Mantel, N. 1967. The detection of disease clustering and a generalised regression approach. *Cancer Research* 27:209–220.
- Martelli, M. 1948. Osservazioni su due species del genere *Larinus* Germ. *Redia* 33:221–286.
- McCoy, K. D., T. Boulmier, C. Tirard, and Y. Michalakis. 2001. Host specificity of a generalist parasite: genetic evidence of sympatric host races in the seabird tick *Ixodes uriae*. *Journal of Evolutionary Biology* 14:395–405.
- McCullagh, P., and J. A. Nelder. 1989. *Generalized Linear Models*. Chapman and Hall, London.
- McKay, J. K., and R. G. Latta. 2002. Adaptive population divergence: markers, QTL and traits. *Trends in Ecology and Evolution* 17:285–291.
- McPheron, B. A., D. C. Smith, and S. H. Berlocher. 1988. Genetic differences between host races of *Rhagoletis pomonella*. *Nature* 336:64–66.
- Messina, F. J. 2004. How labile are the egg-laying preferences of seed beetles? *Ecological Entomology* 29:318–326.
- Michalakis, Y., D. T. Brieese, and A. W. Sheppard. 1992. The taxonomic status of *Larinus cynarae* F. and *Larinus latus* Herbst (Coleoptera: Curculionidae), and its implications for the biological control of *Onopordum* (Asteraceae: cardueae) in Australia. *Biocontrol Science and Technology* 2:275–280.
- Michalakis, Y., A. W. Sheppard, V. Noël, and I. Olivieri. 1993. Population structure of a herbivorous insect and its host plant on a microgeographic scale. *Evolution* 47:1611–1616.
- Noor, M. A. F. 1999. Reinforcement and other consequences of sympatry. *Heredity* 83:503–508.
- Nosil, P., B. J. Crespi, and C. P. Sandoval. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. *Nature* 417:440–443.
- Nosil, P., B. J. Crespi, R. Gries, and G. Gries. 2007. Natural selection and divergence in mate preference during speciation. *Genetica* 129:309–327.
- Ochsmann, J. 1999. Chromosomenzahlen einiger europäischer *Centaurea*-Sippen (Asteraceae). *Haussknechtia* 7:59–65.
- Petney, T. N. 1988. Influence of insect attack on reproductive potential of thistle species in Jordan. *Entomologia Generalis* 14:25–35.
- Pinheiro, J. C., and D. M. Bates. 2000. *Mixed-Effects Models in S and S-Plus*. Springer, New York.
- Raymond, M., and F. Rousset. 1995. GENEPOP (ver. 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86:248–249.
- Rice, W. R. 1985. Disruptive selection on habitat preference and the evolution of reproductive isolation: an exploratory experiment. *Evolution* 39:645–656.
- Russell, F. L., and S. M. Louda. 2005. Indirect interaction between two native thistles mediated by an invasive exotic floral herbivore. *Oecologia* 146:373–384.
- Russell, F. L., S. M. Louda, T. A. Rand, and S. D. Kachman. 2007. Variation in herbivore-mediated indirect effects of an invasive plant on a native plant. *Ecology* 88:413–423.
- Sheppard, A. W., R. D. van Klinken, and T. A. Heard. 2005. Scientific advances in the analysis of direct risks of weed biological control agents to nontarget plants. *Biological Control* 35:215–226.
- Singer, M. C. 1971. Evolution of food plant preference in the butterfly *Euphydryas editha*. *Evolution* 25:383–387.
- Singer, M. C. 2004. Oviposition preference: its definition, measurement, correlates and its use in assessing risks of host shifts. In J. M. Cullen et al., eds. *Proceedings of the XI International Symposium on Biological Control of Weeds*, pp. 235–244. CSIRO Entomology, Canberra.
- Singer, M. C., and C. Parmesan. 1993. Sources of variations in patterns of plant–insect association. *Nature* 361:251–253.
- Singer, M. C., D. Ng, and C. D. Thomas. 1988. Heritability of oviposition preference and its relationship to offspring

- performance within a single insect population. *Evolution* **42**:977–985.
- Singer, M. C., C. D. Thomas, and C. Parmesan. 1993. Rapid human-induced evolution of insect–host associations. *Nature* **366**:681–683.
- Sobhian, R., and L. Fornasari. 1994. Biology of *Larinus curtus* Hochhut (Coleoptera: Curculionidae), a European weevil for biological control of yellow starthistle, *Centaurea solstitialis* L. (Asteraceae), in the United States. *Biological Control* **4**:328–335.
- Strong, D. R. 1997. Fear no weevil? *Science* **277**:1058–1059.
- Taber, S. W. 1994. Labile behavioral evolution in a genus of agricultural pests: the *Rhopalosiphum* plant lice (Hemiptera: Aphididae). *Annals of the Entomological Society of America* **87**:311–320.
- Thompson, J. N. 1993. Preference hierarchies and the origin of geographic specialization in host use in swallowtail butterflies. *Evolution* **47**:1585–1594.
- Thompson, J. N. 1994. *The Coevolutionary Process*. University of Chicago Press, Chicago, IL.
- Tucic, N., and D. Seslija. 2007. Genetic architecture of differences in oviposition preference between ancestral and derived populations of the seed beetle *Acanthoscelides obtectus*. *Heredity* **98**:268–273.
- Via, S. 1999. Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. *Evolution* **53**:1446–1457.
- Via, S. 2001. Sympatric speciation in animals: the ugly duckling grows up. *Trends in Ecology and Evolution* **16**:381–390.
- Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* **38**:1358–1370.
- Wilson, J. R. U., O. Ajuonu, T. D. Center, M. P. Hill, M. H. Julien, F. F. Katagira, P. Neuenschwander *et al.* 2007. The decline of water hyacinth on Lake Victoria was due to biological control by *Neochitina* spp. *Aquatic Botany* **87**:90–93.
- Wright, S. 1951. The genetical structure of populations. *Annals of Eugenics* **15**:323–354.
- Wright, S. 1969. *Evolution and the Genetics of Populations. Vol. 2. The Theory of Gene Frequencies*. The University of Chicago Press, Chicago, IL.
- Xie, X. F., J. Rull, A. P. Michel, S. Velez, A. A. Forbes, N. F. Lobo, M. Aluja, and J. L. Feder. 2007. Hawthorn-infesting populations of *Rhagoletis pomonella* in Mexico and speciation mode plurality. *Evolution* **61**:1091–1105.
- Zwölfer, H., and J. Herbst. 1988. Präadaptation, wirtskreiserweiterung und parallel-cladogenese in der evolution von phytophagen insekten. *Zeitschrift für Zoologische Systematik und Evolutionforschung* **26**:320–340.

## Appendix

Allele frequencies in each population for each of 10 allozyme loci in Sardinia and Corsica). *n*, number of individuals sampled per population and locus (number of genes sampled = 2*n*). Populations CS20, MS16, CS8, and CS12 : weevils were sampled from *Cynara* flowerheads in Sardinia; OS4, OS2, OS21, OS14 :weevils were sampled from *Onopordum* flowerheads in Sardinia; OA22 and OA23 : weevils were sampled from *Onopordum* flowerheads in Corsica.

Loci	Alleles	Populations									
		<i>Cynara cardunculus</i>				<i>Onopordum illyricum</i>					
		CS8	CS12	CM16	CS20	OS2	OS4	OS14	OS21	OA22	OA23
Idh											
( <i>n</i> )		28	41	24	36	28	35	14	16	31	25
	E	0.107	0.110	0.063	0.083	0.125	0.171	0.250	0.031	0.307	0.300
	F	0.000	0.000	0.021	0.000	0.089	0.014	0.000	0.094	0.000	0.000
	G	0.786	0.732	0.771	0.861	0.750	0.729	0.750	0.719	0.532	0.380
	I	0.107	0.159	0.146	0.056	0.036	0.086	0.000	0.156	0.161	0.320
Mdh2											
( <i>n</i> )		28	44	30	37	30	39	14	16	35	33
	B	1.000	1.000	0.983	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	C	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGM											
( <i>n</i> )		28	41	22	36	29	38	13	15	35	28
	A	0.018	0.061	0.046	0.028	0.052	0.013	0.039	0.033	0.029	0.036
	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.029	0.000
	C	0.964	0.939	0.909	0.972	0.948	0.987	0.923	0.967	0.929	0.964
	E	0.018	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.014	0.000
	H	0.000	0.000	0.000	0.000	0.000	0.000	0.039	0.000	0.000	0.000



**Appendix** (Continued)

Loci	Alleles	Populations									
		<i>Cynara cardunculus</i>				<i>Onopordum illyricum</i>					
		CS8	CS12	CM16	CS20	OS2	OS4	OS14	OS21	OA22	OA23
GOT1											
(n)		28	44	29	37	30	39	14	16	35	33
	A	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	0.036	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.015
	C	0.893	0.796	0.810	0.878	0.867	0.859	0.821	0.875	0.957	0.970
	G	0.071	0.182	0.190	0.122	0.133	0.128	0.179	0.125	0.043	0.015
ME											
(n)		28	44	28	37	30	39	14	16	35	33
	A	0.143	0.023	0.000	0.068	0.017	0.013	0.036	0.000	0.000	0.030
	B	0.696	0.932	0.893	0.716	0.967	0.833	0.929	0.969	0.986	0.939
	C	0.161	0.046	0.107	0.216	0.017	0.154	0.036	0.031	0.014	0.030
PGI											
(n)		28	44	30	37	30	39	14	16	35	33
	C	0.000	0.000	0.000	0.027	0.000	0.000	0.107	0.000	0.043	0.000
	D	0.018	0.000	0.067	0.000	0.000	0.000	0.000	0.000	0.014	0.015
	E	0.982	1.000	0.933	0.973	0.983	1.000	0.893	1.000	0.929	0.939
	H	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.046
	I	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000
Sod1											
(n)		28	44	30	37	30	39	14	16	35	33
	A	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.015
	C	1.000	1.000	0.983	1.000	1.000	1.000	1.000	1.000	1.000	0.985
Sod2											
(n)		28	44	30	37	30	39	14	16	35	33
	A	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.063	0.000	0.000
	C	1.000	1.000	1.000	1.000	0.983	1.000	1.000	0.938	1.000	1.000
HK											
(n)		28	38	30	28	30	37	14	16	34	32
	A	0.000	0.040	0.000	0.089	0.067	0.027	0.286	0.188	0.088	0.125
	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.000
	C	0.911	0.908	0.967	0.911	0.850	0.865	0.643	0.781	0.794	0.813
	E	0.089	0.053	0.033	0.000	0.083	0.108	0.071	0.000	0.118	0.063
Mdh1											
(n)		26	30	30	37	28	39	14	15	33	30
	A	0.077	0.050	0.033	0.000	0.000	0.039	0.000	0.000	0.000	0.000
	B	0.000	0.033	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	C	0.885	0.883	0.933	1.000	0.911	0.910	1.000	0.967	0.985	1.000
	D	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.000
	E	0.019	0.033	0.017	0.000	0.089	0.051	0.000	0.033	0.000	0.000