Incorporation of an invasive plant into a native insect herbivore food web

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The integration of invasive species into native food webs represent multifarious dynamics of ecological and evolutionary processes. We document incorporation of *Prunus serotina* (black cherry) into native insect food webs. We find that *P. serotina* harbours a herbivore community less dense but more diverse than its native relative, *P. padus* (bird cherry), with similar proportions of specialists and generalists. While herbivory on *P. padus* remained stable over the past century, that on *P. serotina* gradually doubled. We show that *P. serotina* may have evolved changes in investment in cyanogenic glycosides compared with its native range. In the leaf beetle *Gonioctena quinquepunctata*, recently shifted from native *Sorbus aucuparia* to *P. serotina*, we find divergent host preferences on *Sorbus*- versus *Prunus*-derived populations, and weak host-specific differentiation among 380 individuals genotyped for 119 SNP loci. We conclude that evolutionary processes may generate a specialized herbivore community on an invasive plant, allowing prognoses of reduced invasiveness over time. On the basis of the results presented here, we would like to caution that manual control might have the adverse effect of a slowing down of processes of adaptation, and a delay in the decline of the invasive character of *P. serotina*.

1 [title]

2 **Incorporation of an Invasive Plant**

3 **Into a Native Insect Herbivore Food Web**

4

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21

22 **Abstract**

23   The integration of invasive species into native food webs represent multifarious dynamics

24   of ecological and evolutionary processes. We document incorporation of *Prunus serotina*

25   (black cherry) into native insect food webs. We find that *P. serotina* harbours a herbivore

26   community less dense but more diverse than its native relative, *P. padus* (bird cherry), with

27   similar proportions of specialists and generalists. While herbivory on *P. padus* remained

28   stable over the past century, that on *P. serotina* gradually doubled. We show that *P. serotina*

29   may have evolved changes in investment in cyanogenic glycosides compared with its native

30   range. In the leaf beetle *Gonioctena quinquepunctata*, recently shifted from native *Sorbus*

31   *aucuparia* to *P. serotina*, we find divergent host preferences on *Sorbus*- versus *Prunus*-

32   derived populations, and weak host-specific differentiation among 380 individuals

33   genotyped for 119 SNP loci. We conclude that evolutionary processes may generate a

34   specialized herbivore community on an invasive plant, allowing prognoses of reduced

35   invasiveness over time. On the basis of the results presented here, we would like to caution

36   that manual control might have the adverse effect of a slowing down of processes of

37   adaptation, and a delay in the decline of the invasive character of *P. serotina*.

Introduction

38

39 The introduction and subsequent explosive spread of non-native species is seen as one of

40 the main environmental disturbances threatening ecosystems globally (Glowka *et al.*, 1994;

41 Gurevitch & Padilla, 2004; Simberloff, 2011). Not all introduced species will eventually

42 successfully establish themselves and spread invasively (Williamson & Fitter, 1996). For

43 example, populations of colonists may die out due to disease or adverse environmental

44 conditions (Rodriguez-Cabal *et al.*, 2013). Nonetheless, the numbers of environmentally

45 problematic exotics are increasing worldwide (Butchart *et al.*, 2010). This even holds for

46 parts of the world that are traditionally seen as sources, rather than recipients of exotic

47 species, such as Europe (Hulme *et al.*, 2009; van Kleunen *et al.*, 2015).

48 One potential explanation for the invasiveness of an introduced species is the so-called

49 enemy release hypothesis, ERH (Keane & Crawley, 2002; Liu & Stiling, 2006), which states

50 that, because the introduced species has not coevolved with the native biota, release from

51 specialized parasites and predators causes explosive population growth.

52 Enemy release may cause the initial spread, but the subsequent population dynamics are

53 more complex, and influenced by evolutionary processes. Reduced selection pressure for

54 defences against specialist herbivores may result in the evolution of changed energy

55 investment. For example, the plant may evolve stronger allocation of resources towards

56 growth and reproduction and/or towards defence against generalists (Blossey & Nötzold,

57 1995; Joshi & Vrieling, 2005; Zangerl & Berenbaum, 2005; Prentis *et al.*, 2008; Whitney &

58 Gabler, 2008). However, at the same time, native herbivores may evolve the ability to

59 locate and feed on introduced species (Vellend *et al.*, 2007; Pearse & Hipp, 2014).

60   Therefore, the course of the establishment of an introduced species is complex, with

61   population dynamics modified by evolution: over time, the community of natural enemies

62   attacking an introduced species tends to expand (Brändle *et al.*, 2008) and the adverse

63   impact of invasive species tends to wane (Williamson, 1996; Simberloff & Gibbons, 2004;

64   Blackburn *et al.*, 2009; Dostál *et al.*, 2013). This may be due to evolution in both the invader

65   and the species it interacts with (Vellend *et al.*, 2007). However, a species' invasive

66   character is often considered static, and management policies rarely consider the

67   possibility that it may change due to evolutionary adaptation (Whitney & Gabler, 2008).

68   One prominent invasive plant species in Europe is the black cherry, *Prunus serotina* Ehrh,

69   native of eastern North America and considered a "forest pest" in Europe after widespread

70   planting as auxiliary tree in pine plantations throughout the 20th century (Schütz, 1988;

71   Bakker, 1963; Starfinger *et al.*, 2003). Being bird-dispersed, it has been rapidly invading

72   forested and open habitats (Deckers *et al.*, 2005). In many European countries (Starfinger

73   *et al.*, 2003), it is now considered one of the most important threats to habitat quality of

74   vegetation on dry, acidic, and/or poor soil, such as dunes and moorland (Fig. 1; Godefroid

75   *et al.*, 2005). In the Netherlands, for example, *P. serotina* has increased in distribution and

76   abundance by at least two orders of magnitude during the second half of the 20th century

77   (Tamis *et al.*, 2005). Current control measures (chemical and mechanical eradication) are

78   temporary and cosmetic (Starfinger *et al.*, 2003). Nonetheless, they are costly: Reinhardt *et

79   al.* (2003) conservatively calculated the annual cost of *P. serotina* control in Germany to be

80   ca. 25 million euros.

81   Possibly the initial spread of *P. serotina* was facilitated by an absence of natural enemies;

82 for example, Reinhart *et al.* (2003) found that, in the native range, soil pathogens inhibit the

83 establishment of *P. serotina* seedlings near conspecifics, whereas in the invaded range, the

84 species-specific soil community facilitates establishment. However, it is to be expected that

85 the rich resource which *P. serotina* constitutes will provide adaptive opportunities for

86 phytophagous insects to exploit. Such an evolutionary process will be even more likely if *P.*

87 *serotina* represents an enemy-free space for herbivores (see Feder [1995] and Karolewski

88 *et al.* [2014] for examples in other plants), and if it has been evolving reduced herbivore

89 defences (Blossey & Nötzold, 1995). The changes in chemical defences may be complex.

90 Joshi & Vrieling (2005) found that invasive plants may increase energetically cheap

91 defences aimed at generalist herbivores, while reducing costly defences aimed at

92 specialists when these specialists are no longer present.

93 Reports of native insects exploiting introduced *P. serotina* in Europe have been scarce

94 throughout much of the 20th century, and have mostly concerned accidental feeding (by,

95 e.g., moths, aphids, weevils, and leaf beetles; Korringa, 1947; Hille Ris Lambers, 1971;

96 Moraal, 1988; Klaiber, 1999; Fotopoulos, 2000). Simultaneously, at least among nature

97 management workers, a widespread belief has been maintained that the strong cyanogenic

98 properties of the species, stronger than in *P. padus* (Poulton, 1990; Swain *et al.*, 1991;

99 Santamour, 1998; Hu & Poulton, 1999; Fitzgerald, 2008; Pimenta *et al.*, 2014), have

100 prevented native insect herbivores from colonizing it (Nyssen *et al.*, 2013; Anonymous,

101 2014). More recently, however, studies from France, Germany, the Netherlands, and Poland

102 are beginning to suggest that a community of native herbivores may in fact be

103 accumulating on *P. serotina* (Karolewski *et al.*, 2014; Wimmer & Winkel, 2000; Winkelman,

104 2005; Nowakowska & Halarewicz, 2006; Żmuda *et al.*, 2008; Boucault, 2009; Halarewicz &

105   Jackowski, 2011; Meijer *et al.*, 2012; Karolewski *et al.*, 2013).

106   In this paper, we investigate the composition of the insect herbivore community feeding on

107   *P. serotina* in the Netherlands. Because congenerics are likely to have been an important

108   source of colonists, we compare the *P. serotina* herbivore community with the one

109   occurring locally on *P. padus*, its closest native relative in the Netherlands (Bortiri *et al.*,

110   2001). To obtain an impression of the accumulation of herbivory in *P. serotina*, herbivore

111   damage in both *Prunus* species is quantified on the basis of herbarium records. We then

112   investigate the impact that two conditions may have had on herbivore presence:

113   cyanogenic defence compounds and parasitoid attack, in both *Prunus* species. Finally, as an

114   example of the adaptive evolution that specialist *P. serotina* herbivores may have

115   undergone, we studied host preference and genomics in one particular *P. serotina*

116   herbivore, the leaf beetle *Gonioctena quinquepunctata*.

117

Materials and Methods

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Sampling herbivore communities on *P. serotina* and *P. padus*

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122 The insect community feeding on both *Prunus* species was sampled in Nationaal Park Zuid-

123 Kennemerland (52° 25′ N, 4° 35′ E), a partly forested area of coastal sand dunes near

124 Haarlem, the Netherlands. Sampling was done by traversing a 2 x 2 km area in the old,

125 forested dunes, and haphazardly selecting 300 individuals (150 of each species). We took

126 care that on each day, roughly equal numbers of *P. padus* and *P. serotina* were investigated.

127 Where possible, individuals of the two species were sampled in alternation. Sampling was

128 done manually (no tools like nets, beating trays, or exhausters were used) in spring and

129 early summer of 2009 (3 days), 2010 (10 days), and 2012 (8 days), by a single person

130 inspecting, for 5 min., leaves, twigs, flowers, and fruits up to c. 2.5 m above ground level. All

131 insects feeding or ovipositing on the host plant were stored in 96% ethanol. To obtain

132 measurements on the actual amount of foliage searched, we replicated the above sampling

133 method in September 2015 on 10 and 8 trees, respectively, of *P. serotina* and *P. padus*, and

134 counted the numbers of leaves and lengths of twigs searched. We also determined fresh

135 weights of ten leaves of each of the two plant species. Insects were identified

136 morphologically, with help from experts (see Acknowledgements). The 2009 and 2010

137 Geometridae and Tortricidae were identified by sequencing of the Cytochrome Oxidase I

138 DNA-barcode region (e.g., Van Nieukerken *et al.*, 2011) and the "animal identification"

139 module in BOLD (www.boldsystems.org). All 2009 and 2010 specimens were deposited in

140 the collections of Naturalis Biodiversity Center (container codes BE90711-90716). Because

141   of improper curation, the specimens from the 2012 sampling were discarded after

142   identification. We adopted Leather's (Leather, 1985) host range indicators of G (generalist,

143   feeding on multiple plant families), R (feeding on Rosaceae only), P (on *Prunus* only), and M

144   (monophagous, feeding on *P. padus* only). In addition, we categorized species that are

145   specialized on non-Rosaceae (e.g., *Quercus*-specialists) as O ("other"). Differences in species

146   richness for each of these categories were compared between both host species and tested

147   for significance with a chi-square test. Natuurmonumenten (Ruud Luntz) permitted us to

148   work in Nationaal Park Zuid-Kennemerland under permit No. 19 of 2008. Dunea (Harrie

149   van der Hagen) permitted us to work in Meijendel by permission 25/2/2013.

150

151   Herbivory history on *Prunus padus* and *Prunus serotina*

152

153   We used historical accessions of *P. padus* and *P. serotina* in the herbarium collection of

154   Naturalis Biodiversity Center to produce time-series of insect herbivory in the Netherlands

155   for both hosts. Herbivory was assessed by a method of our own design, as percentage of

156   leaves on a herbarium specimen that showed pre-collection insect damage (post-collection

157   damage by herbarium beetles was recognized and recorded, but not included in the

158   herbivory data). We are aware of the fact that some botanists may preferentially have

159   collected undamaged branches, so these estimates of herbivory are to be treated as

160   conservative. We assessed changes of herbivory over time by Pearson tests on linear

161   correlation coefficients.

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164   Parasitization of caterpillars

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166   Within the same 2 x 2 km area as mentioned above, we sampled 173 and 110 live

167   caterpillars from 43 *P. padus* and 32 *P. serotina* trees, respectively, between May 18th and

168   June 3rd, 2011. All caterpillars were reared in individual vials. If a caterpillar

169   metamorphosed into an adult moth or butterfly, it was considered unparasitized. If a

170   parasitoid wasp or fly emerged, the host was considered parasitized. Caterpillars or pupae

171   from which no adult insect had emerged by June 19th, were dissected in ethanol or Ringer's

172   solution to determine the presence or absence of parasitoid eggs, larvae, pupae, or adults

173   (Zchori-Fein *et al.*, 2001). When found, these hosts were also considered as parasitized.

174   Models describing the binominal response variable "parasitized" (Y/N) with combinations

175   and interactions of the following explanatory variables: tree, method, xylosteana, and tree-

176   ID (which was added as a random effect) were created and analysed in R 2.12.1 (R

177   Development Core Team, 2010). "Tree" was the caterpillar's host plant species (*P. padus* /

178   *P. serotina*). "Method" was the way a caterpillar was determined to have been parasitized

179   or not (dissected in ethanol, dissected in Ringer's solution, or reared to adult or parasitoid

180   emergence). "Xylosteana" indicated if the caterpillars belonged to the most commonly

181   encountered species, *Archips xylosteana* (TRUE) or another species (FALSE). Of the

182   identified caterpillars, all other species were not present in sufficient numbers (<8) for

183   species-level analysis.

184

185

186   Determination of cyanogenic glycosides

187

188   We analysed secondary plant compounds for 57 of the *P. padus* and 56 of the *P. serotina*

189   plants for which we sampled herbivores in 2012 (see above). Immediately after each

190   herbivore sampling, we harvested five young leaves and five old leaves from each tree, and

191   kept these in separately labelled bags in a Dewar flask with solid CO_2 in the field. All

192   samples were ground under liquid nitrogen and freeze-dried. We carried out NMR-analysis

193   as described previously (Pimenta *et al*., 2014; Kim *et al*., 2003; Kim *et al*., 2010). Briefly,

194   extracts in CH_3OH-d4 and KH_2PO_4 buffer in D_2O (1:1) were quantitatively analysed for

195   prunasin and amygdalin, using 1H-NMR spectroscopy on a 500MHz Bruker DMX-500

196   spectrometer (Bruker, Karlsruhe, Germany). Purity of quantitated 1H-NMR signals was

197   evaluated using several two-dimensional NMR experiments. Correlations were investigated

198   between concentrations of each of the cyanogenic glycosides and herbivore load. We

199   treated generalists (category G, see above) and specialists (categories R, P, M, and O)

200   separately. In view of the high numbers of *Yponomeuta evonymellus* and *Rhopalosiphum*

201   *padi* on some *P. padus* trees, we log-transformed the specialist herbivore load for *P. padus*.

202   The relative amounts of cyanogenic glycosides were calculated per sample by taking the

203   integrals in buckets δ 5.92 (for prunasin) and δ 5. 88 + δ 5.84 (for amygdalin). Correlations

204   were tested with parametric Pearson's tests for the data on generalists and (in view of the

205   large numbers of samples devoid of specialists) with non-parametric Spearman's tests for

206   the data on specialists.

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209   A specialist herbivore's food preference for the original *Sorbus* vs. the novel

210   *Prunus serotina*

211

212   We selected the oligophagous leaf beetle *G. quinquepunctata* for a case study of host

213   preference. We chose this species because (i) it has very recently (probably in the early

214   1990s) colonized *P. serotina* in north-central Europe (Klaiber, 1999; Halarewicz &

215   Jackowski, 2011; Meijer *et al.*, 2012; Mazderek *et al.*, 2015); (ii) it is a specialized species,

216   originally feeding chiefly on rowan, *Sorbus aucuparia* (Wimmer & Winkel, 2000; Koch,

217   1992). Within a circle with 6-km radius around Eelde (53° 08′ N, 6° 34′ E), this beetle only

218   feeds on the original native host *S. aucuparia* and the novel introduced *P. serotina* (not on

219   any other hosts), and is equally abundant on both (Meijer, 2013). In May 2011, 83 adults

220   and 138 larvae were collected from *S. aucuparia* and 63 adults and 57 larvae were collected

221   from *P. serotina*, and kept separate by collection locality and host plant. These were used in

222   host choice experiments: one *S. aucuparia* and one *P. serotina* branch (with 3-5 leaves each)

223   was placed in a bottle filled with water, which was then placed in the centre of a 0.25 m^3

224   cage. Between one and five adults or between two and 10 larvae were selected from one of

225   the live, host-specific collections and placed on the plug in the neck of the bottle. Each

226   experiment was conducted with individuals from only one of the two hosts, and each

227   individual was tested only once. Adults and larvae were not mixed within an experiment.

228   After 21 h, the position for each individual was recorded and the animals were returned to

229   their respective live collections. The test was performed 107 times. Tests were carried out

230   on animals collected within a two-week period and were begun on the date that they were

231   collected. We then tested for host preference using a GLM with binomial distribution. The

232   model included the fixed factors of original host plant, life stage (larva or adult), interaction

233   between original host plant and life stage, collection date, locality of origin, and cage

234   (multiple cages were used). The effect of each factor was tested by removing one factor and

235   comparing the complete model with the reduced model, and to do this successively with

236   each of the factors, using ANOVA. Host preference in *G. quinquepunctata* was tested with a

237   proportion test, by comparing the host choices for all animals, depending on their host of

238   origin. All analyses were done in R (R Development Core Team, 2010).

239

240

241   Genomic differentiation in host-specific subpopulations of a specialist

242   herbivore

243

244   Using the same *G. quinquepunctata* specimens from Eelde as above, after finishing the host

245   choice tests, we chose one adult individual from each host plant and obtained full genome

246   sequences from these using paired-end forward-reverse sequencing on an Illumina HiSeq

247   2000. We pooled the data from both *G. quinquepunctata* sequencing runs and used this for

248   a single *de novo* assembly. We assembled the data using Abyss (Simpson *et al.*, 2009) with a

249   *k*-mer length of 23 and a *k*-mer coverage of 3, values which we optimized using KmerGenie

250   (Chikhi & Medvedev, 2013). We saved all produced contigs longer than 200bp. We then

251   mapped the data from both samples separately against these contigs using BWA (Li &

252   Durbin, 2009) at default settings and used Samtools (Li *et al.*, 2009) to call the SNPs in the

253   BWA alignments. We looked up the SNP positions in the alignments for both samples and

254 filtered based on the following criteria: the positions were both homozygous for different

255 alleles between the samples, had a coverage of at least 10x in each sample, had flanking

256 regions that were at least 100bp long with a minimum combined coverage of at least 15x

257 with a maximum of 2 heterozygous positions. We identified the contigs containing valid

258 SNP positions by BLASTing them against the GenBank nucleotide database and removing

259 all non-arthropod contigs. Based on the remaining SNPs, we made a random selection of

260 128 SNPs (Table S5), all from different contigs, for which we designed primers using the

261 Kraken software (LGCgenomics). Subsequently, in June 2014, again within the same 6-km

262 radius around Eelde, we collected a new set of individuals from both hosts at five localities

263 (Norg-1, Norg-2, Kleibos, Appelbergen, and Noordlaarderbos); 206 from *S. aucuparia*, and

264 173 from *P. serotina*. We performed DNA extractions on head+thorax using the NucleoMag

265 96 Tissue kit (Macherey-Nagel Gmbh & Co., Düren, Germany) on the KingFisher Flex

266 magnetic particle processor (Thermo Scientific). DNA was diluted to 1 ng/µl and analysed

267 in uniplex on the LGC Genomics SNP-genotyping line according to manufacturer's

268 instructions. SNPs were detected using the KASP technique (Semagn *et al.*, 2014).

269 Genotypes were called using the Kraken software. We discarded five loci that did not yield

270 scorable SNP-patterns and four loci that deviated from Hardy-Weinberg equilibrium,

271 leaving 119 loci. Missing data were scattered over loci and samples and amounted to 2.9%

272 of the total data set. We assessed population differentiation by Analysis of Molecular

273 Variance (AMOVA), as well as by a Structure analysis (Pritchard *et al.*, 2000; Excoffier &

274 Lischer, 2010). For Structure, standard settings were used and 10 replicates were

275 performed for K=2 to K=10. The results were uploaded to Structure Harvester and a delta K

276 plot was used to determine the number of groups (Earl & vonHoldt, 2012). We used a

277   hierarchical AMOVA with host plants nested within localities, and we repeated the same

278   AMOVA on a locus-by-locus basis.

279

Results

Sampling herbivore communities on *Prunus serotina* and *Prunus padus*

Our sampling method covered on average, per tree, 258 (± 136 s.d.) and 141 (± 91 s.d.)
leaves of *P. serotina* and *P. padus*, respectively. Given mean fresh weights of *P. serotina* and
P. padus leaves of 0.44 and 0.91 g, respectively, the amounts of foliage searched in 5
minutes were 113.5 g and 128.3 g for *P. serotina* and *P. padus*, respectively. After correction
for the 1.13 x more foliage searched in *P. padus*, we found that *P. serotina* harbors a 4.15-
fold lower density but almost two-fold higher species diversity of herbivorous insects
(Table 1; Table S1) than *P. padus*. The higher herbivore load on *P. padus* is, however, largely
due to only two monophagous species, *Y. evonymella* (Lepidoptera: Yponomeutidae) and *R.*
padi (Hemiptera: Aphididae), which usually occur in dense "nests" and "colonies",
respectively (Leather, 1985). These two species were found on *P. serotina* at much lower
densities and usually only as single individuals. Almost half of the herbivore specimens
found on *P. padus* belong to these two species. We did not find a difference in the
proportions of specialists versus generalists on the native and the non-native host (Fig. 2):
both species carried similar (chi-square = 4.13; *P* = 0.38) proportions of each of the four
categories of host range (G, generalists; R, Rosaceae-specialists; P, *Prunus*-specialists; M, *P.*
padus monophages; and O, other—mostly *Quercus*—specialists).

302   History of herbivory on *Prunus padus* and *Prunus serotina*

303

304   Herbarium records (Table S2) for *P. serotina* (n = 96; 2817 leaves) showed a more than

305   two-fold increase in herbivory (proportion damaged leaves) from 18.8% to 40.6% over the

306   past 170 years (r = 0.262; *P* = 0.0099, df = 94; Pearson test; Fig. 3A). For *P. padus* (n = 222;

307   6612 leaves), herbivory has remained stable at c. 35% over the past two centuries (r = -

308   0.020; *P* = 0.766, Pearson test; Fig. 3B). In the most recent year (2013) we found no

309   significant difference between the herbivory in *P. padus* (40%) and *P. serotina* (41%) (T-

310   test; *P* = 0.53).

311

312

313   Parasitization of caterpillars

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315   The percentages of parasitized caterpillars on both *Prunus* species were not significantly

316   different (*P. padus*: 55/173, 32%; *P. serotina*: 43/110, 39%; chi-square = 1.58; *P* = 0.21).

317   Tables of explanatory variables and response variables are presented in Table S8. A third of

318   all collected specimens belonged to *Archips xylosteana*. A test of independence of the

319   explanatory variable tree explaining the response variable "parasitized" was not significant

320   (chi-square = 1.58, df = 1, *P* = 0.20). A full generalized linear model was used to described

321   the response variable "parasitized" as a three-way interaction between "tree", "method",

322   and "xylosteana". The full model was not significant, and after simplifying the model by

323   steps, the only explanatory variable to affect parasitization significantly was the method

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324   used to determine if a specimen was infected by a parasitoid ($P < 0.01$). The identified

325   parasitoids mostly belonged to Ichneumonidae, Braconidae, and Tachinidae.

326

327

328   Determination of cyanogenic glycosides

329

330   In the NMR-analyses (Table S3), we found that the concentration of cyanogenic glycosides

331   (prunasin and amygdalin combined) per unit leaf dry weight is similar in both *Prunus*

332   species. Mean concentrations in young and old leaves differed by < 5% in each plant

333   species. In both plant species, the ratio prunasin : amygdalin was c. 3 : 1. Generalist and

334   specialist herbivores showed different relations with cyanogenic glycoside concentrations,

335   and the responses to prunasin differed from those to amygdalin. Specifically, we found that

336   the generalist herbivore load was not correlated with prunasin (R = -0.08, P = 0.39, both in

337   *P. prunus* and *P. serotina*), but increased with amygdalin concentration (R = 0.24 and 0.36;

338   P = 0.01 and 0.0001, respectively, in *P. padus* and *P. serotina*), whereas the specialist

339   herbivore load increased with prunasin concentration, and decreased with amygdalin

340   concentration, but significantly so only in *P. padus* (of which the amygdalin relationship

341   would lose significance after Bonferroni correction; see statistical test results given in Fig.

342   4).

343

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345 A specialist herbivore's food preference for the original *Sorbus* vs. the novel

346 *Prunus serotina*

347

348 At the end of the host choice experiment, 52% of all experimental *G. quinquepunctata* were

349 present on one of the host plants. Individuals collected on *S. aucuparia* showed a significant

350 preference for *S. aucuparia* (69.7 ± 3.1%) over *P. serotina* ($P < 0.0001$). However,

351 individuals collected on *P. serotina* showed no significant preference for either host. Similar

352 patterns were found in both adults and larvae: Individuals from *S. aucuparia* preferred

353 their original host (75.9 ±7.0 % for adults, $P < 0.0001$, and 65.9 ± 9.0 % for larvae, $P =$

354 0.0003); individuals from *P. serotina* showed no preference (58.7 ± 9.1 % for adults, $P =$

355 0.2077, and 57.9 ± 14.3 % for larvae, $P = 0.2893$). Full test results are available in Table S7.

356

357

358 Genomic differentiation in host-specific subpopulations of a specialist

359 herbivore

360

361 Illumina sequencing of a *G. quinquepunctata* larva from *S. aucuparia* gave 157,327,896

362 reads, and 191,340,606 reads were obtained from an adult beetle found on *P. serotina*. The

363 de-novo assembly with Abyss resulted in 438,237 contigs longer than 200 bp. The data

364 were deposited in the NCBI short read archive under BioProject accession code:

365 PRJNA277307. A total of 729 usable SNPs were obtained from the SNP discovery. To assess

366 genetic differentiation in both host-specific subpopulations, we genotyped 379 individuals

367   from both hosts at each of five localities, for the selected 119 SNP loci (Table S4). Our

368   Structure analysis (SI Text S1) failed to detect overall genetic differentiation between the

369   populations on both host plants: the highest posterior probability was for K = 2, but these

370   two groups did not correspond with host plant nor with locality. The hierarchical AMOVA

371   with host plants nested within localities, showed significant ($P < 0.01$) differentiation

372   between host plants in each locality. On a locus-by-locus basis, the AMOVA revealed 13 loci

373   that were significantly differentiated between the two subpopulations from *P. serotina* and

374   *S. aucuparia*, two of which remained significant after strict Bonferroni correction (Table

375   S6). The distribution of per-locus pairwise (*Prunus*-*Sorbus*) F_{ST} values (Fig. 5) also shows

376   that at least two loci are outliers. Homology searches in Genbank for these SNP loci yielded

377   no matches with genes of known function.

378

Discussion

379

380

381 Our inventories show that the invasive *P. serotina* in the Netherlands harbours a

382 surprisingly rich community of herbivores. Although the densities were lower than on

383 native *P. padus*, the species diversity was greater. Also, contrary to expectations, the *P.*

384 *serotina* herbivore community contained similar proportions of specialists versus

385 generalists as the one on *P. padus*. The only species strikingly absent from *P. serotina* were

386 two abundant *P. padus* monophages, *Y. evonymellus* and *R. padi*. Consistent with Leather

387 (1985), both species were responsible for more than two thirds of all insects found feeding

388 on *P. padus*, whereas they occurred on *P. serotina* only in small numbers (we found only a

389 single *Y. evonymellus* caterpillar and a single *R. padi* colony on *P. serotina*). Nonetheless,

390 laboratory data (Kooi *et al.*, 1991) and field data from Poland (Karolewski *et al.*, 2014)

391 suggest that at least *Y. evonymellus* has the potential to feed on *P. serotina*. Karolewski *et al.*

392 (2014) state that in Poland, the latter species has progressed from avoiding *P. serotina*

393 altogether to feeding and developing on it massively over the past decade. The near-

394 absence from *P. serotina* of this herbivore in our study area suggests that a similar

395 colonization event may not yet have taken place, but this may change in the near future,

396 possibly aided by long-distance gene flow from the populations in Poland. Another striking

397 difference between both hosts is the relatively large numbers of non-Rosaceae specialists

398 on *P. serotina*. While some of these may be accidental "tourists", the high number of

399 individuals for some of these species (e.g., the *Quercus*-specialist *Harpocera thoracica*) is

400 noteworthy.

401   These results add to a body of data on insect herbivory on native versus non-native plants

402   (reviewed in, e.g., Liu & Stiling, 2006; Colautti *et al.*, 2004; Meijer, 2013). Although these

403   studies tend to show that introduced plant species, especially those with powerful chemical

404   defences, are poor hosts for native herbivores, exceptions have also been found of

405   introduced species hosting a larger number of species than closely-related native plants

406   (Novotny *et al.*, 2003). The rich herbivore community on non-native *P. serotina*, and

407   especially the high number of specialist species, fits with the observation that the food web

408   supported by a non-native plant expands as time since initial introduction increases

409   (Brändle *et al.*, 2008). Although *P. serotina* was introduced into Europe earlier (Schütz,

410   1988), it only became common in the 20th century (Starfinger *et al.*, 2003). Its increasing

411   abundance in Dutch ecosystems over the past 80 years may have been the phase during

412   which most of the herbivore community has built up. Indeed, while our study of leaf

413   damage in herbarium specimens cannot reveal the diversity of herbivores, it does show

414   that herbivore damage, and therefore perhaps herbivore load, has gradually doubled over

415   this period, while that on *P. padus* has not changed. Today, at least based on our herbarium

416   records, herbivory levels in both plant species appear to be similar (despite the lower

417   herbivore load that we found in our inventory for *P. serotina*—see above).

418   In theory, the rapid assembly of this community may have been aided by the presence of an

419   enemy-free space for the insect herbivores. If local parasitoids, for example, are not

420   adapted to using *P. serotina* volatiles as a cue for attraction to a possible patch in which to

421   find hosts, this may have helped the establishment of herbivore populations on the

422   introduced plant (Feder, 1995; Harvey & Fortuna, 2012). Indeed, Karolewski *et al.* (2014)

423   found reduced parasitization of one species, *Y. evonymella* on *P. serotina*. However, we find

424   that current attack rates of caterpillars by parasitoids do not differ between *P. padus* and *P.*

425   *serotina*.

426   After an initial period of reduced specialist herbivory in the non-native range, *P. serotina*

427   may have shifted its investment in chemical defences in favour of those aimed at

428   generalists (Joshi & Vrieling, 2005). Cyanogenic glycosides are generally considered to be

429   systemic, non-inducible, and energetically cheap chemical defences aimed primarily at

430   generalist herbivores (Gleadow & Møller, 2014). However, our phytochemical data suggest

431   that, in *P. padus* (and, less clearly, in *P. serotina*), the Rosaceae-specific compound

432   amygdalin has a positive relationship with generalist load but a negative one with specialist

433   load, whereas the more widespread compound prunasin has a positive correlation with

434   specialist herbivore load, while lacking any clear relation with generalist load. It would be

435   tempting to compare the levels and ratios of prunasin and amygdalin in today's *P. serotina*

436   populations in the Netherlands with those reported for the native American population.

437   However, we only have access to a single American study (Santamour, 1998), which,

438   moreover, employed somewhat different methods (see below), so we do so with

439   considerable hesitation. Santamour (1998) reported a summertime HCN production in

440   native American *P. serotina* corresponding to 29.6 mg cyanogenic glycosides per g fresh

441   leaf material (see SI Text S2). In an earlier study of 22 Dutch *P. serotina* trees (Pimenta *et*

442   *al.*, 2014), we found on average 30.4 mg cyanogenic glycosides per mg *dry* leaf material. As

443   *P. serotina* dry leaf weight is 36% of fresh leaf weight (see SI Text S2), this might suggest

444   that total cyanogenic glycoside content in the invaded range could be about two- to

445   threefold lower than in North America. Also, Santamour found prunasin : amygdalin

446   proportions of 22 : 1, whereas we found a ratio of 3 : 1. In the Dutch *P. serotina*, prunasin

447   investment might therefore have decreased, with amygdalin content remaining more or

448   less constant. Since both the absolute and relative amounts of prunasin and amygdalin

449   content have a genetic basis (Santamour, 1998), these results might indicate that

450   cyanogenic glycoside defence has, after the introduction into Europe, adapted to the novel

451   herbivore communities. With a mean age at first reproduction of only 5.2 years (Deckers *et*

452   *al.*, 2005) and evidence, in general, of rapid evolution of defence in invasive plants (Felker-

453   Quinn *et al.*, 2013), such a quick evolutionary change is not implausible. However, since

454   Santamour (1998), Pimenta *et al.* (2014) and the present study appear to be the only

455   available quantifications of prunasin and amygdalin in *P. serotina*, and since the range of

456   phenotypic plasticity in cyanogenic glycoside content is unknown, more data, with more

457   comparable methods, are needed before this conclusion can be substantiated. Moreover,

458   we stress that our results and their discussion refer only to the cyanogenic potential

459   (HCNp), whereas the true defence potential is a combination of HCNp and HCNc,

460   cyanogenic capacity, which is a function of glucosidase presence and activity. Since the

461   latter is unknown in this study, we implicitly assume that HCNp is an indicator for

462   cyanogenic defence, which may only be partly true and is known to differ between

463   specialists and generalists (Ballhorn *et al.* 2010a).

464   The accumulation of the herbivore community on *P. serotina* may also have involved

465   evolutionary processes within the insect community itself. One possibility is that all

466   present-day herbivores were able to feed and reproduce on *P. serotina* from the moment

467   the new host was introduced. However, this would not explain the *slow* increase in

468   herbivory that our herbarium data show: highly mobile insects with short generation times

469   would have established on the new host instantaneously, rather than gradually. It is

470   therefore likely that adaptive evolution in the herbivores played an important role in the

471   assembly of this community over time.

472   As a possible example of this scenario, we performed a case study on one specialist

473   herbivore, the leaf beetle *G. quinquepunctata*, which has recently colonized *P. serotina* from

474   its original host, rowan (*S. aucuparia*). We find indications of weak differentiation in host

475   preference and SNP-loci on *Sorbus*- versus *Prunus*-derived beetle individuals. We found

476   that individuals collected on *Sorbus* retained a significant host preference for this host,

477   whereas beetles collected from *Prunus* showed no preference for *Prunus* over *Sorbus*. We

478   found the same host preference in adults and larvae, although presumably host choice is

479   made mostly in the mobile, adult stage. While these results do not necessarily imply genetic

480   differentiation, as learning may be involved as well (Salloum *et al.*, 2011), our SNP-analysis

481   does show indications of weak genetic differentiation, with several loci showing

482   divergence, and potentially linked to regions that are under disruptive, host-imposed

483   selection. In other words, the introduced *P. serotina* may have selected for weak, incipient

484   divergence (Vellend *et al.*, 2007; Nosil & Feder, 2011) in this particular herbivore. Whether

485   such selection will allow further sympatric speciation, in this herbivore or others, depends

486   not only on the different selection regimes imposed by the different host plants, but also on

487   the mount of gene-flow between the populations feeding on the two hosts (Nosil & Feder,

488   2011).

489   Overall, our results indicate that, since its introduction, a rich and diverse herbivore

490   community has accumulated on *P. serotina*. It is possible that evolutionary adaptations in

491   these herbivores as well as in the plant itself have played an important role in shaping this

492   community. Adaptation may have involved niche widening in generalist herbivores,

493   incipient genetic divergence in specialists, as well as adjustments of chemical defences in

494   the host plant.

495

496   These results may have implications for invasive species management. It may be expected

497   that the gradual evolutionary integration of a novel plant species in a native herbivore food

498   web may eventually reduce its invasive character to the point where it attains the status of

499   non-harmful, naturalized neophyte. Whether this will happen in the case of *P. serotina*

500   depends on a number of factors. In this paper, we dealt with herbivorous insects only,

501   whereas plant demographics are affected by a much broader spectrum of natural enemies.

502   Reinhart *et al*. (2003) and Van der Putten (2000) suggested that its invasiveness may be

503   more due to an absence of belowground interactions (with the *Prunus*-pathogenic fungus

504   *Pythium*, for example) than aboveground interactions. However, preliminary studies in the

505   Netherlands indicate the presence of local *Pythium* populations that are powerful in

506   attacking introduced *P. serotina* (Tamis & van der Klugt, pers. comm.). Furthermore,

507   Ballhorn *et al.* (2010b) and Ballhorn (2011) found that in cyanogenic plants a trade-off

508   exists between defence against herbivores and against fungal pathogens, which is an

509   additional complication not yet considered. A final point of concern is the intensity of the

510   regime of natural selection. Presently, manual control of mature *P. serotina* in many

511   European habitats is reducing the continued exposure of the host to its potential

512   herbivores. On the basis of the results presented here, we would like to caution that this

513   might have the adverse effect of a consequent slowing down of processes of adaptation,

514   and a delay in the decline of the invasive character of *P. serotina*.

515

516 **Acknowledgments**

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531

532 **Competing Interests**

533 Marco Flohil is an employee of ServiceXS, a company providing DNA services such as

534 reported in this paper.

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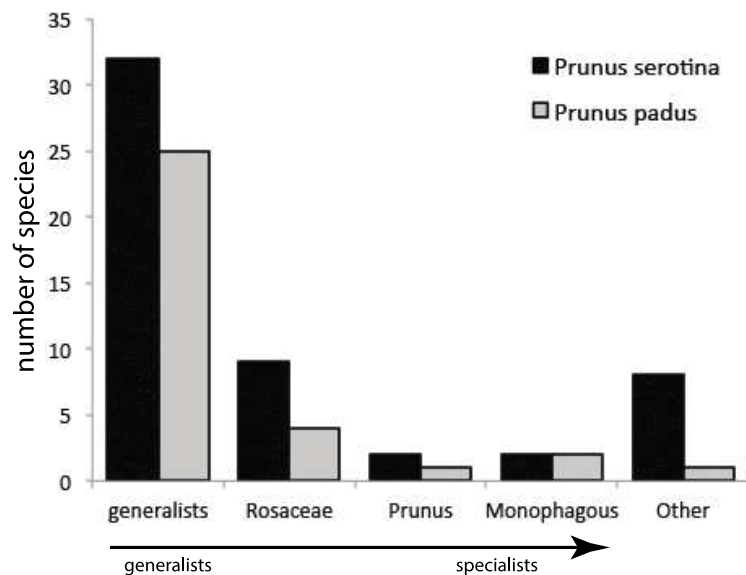
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761 **Figures**

762

763

764 Figure 1. In open habitats, such as this moorland in the Netherlands, *Prunus serotina* may

765 spread invasively, as this carpet of seedlings shows. (photo copyright: Kritisch Bosbeheer).

766

767   Figure 2. Numbers of species from different categories of generalist and specialist insect
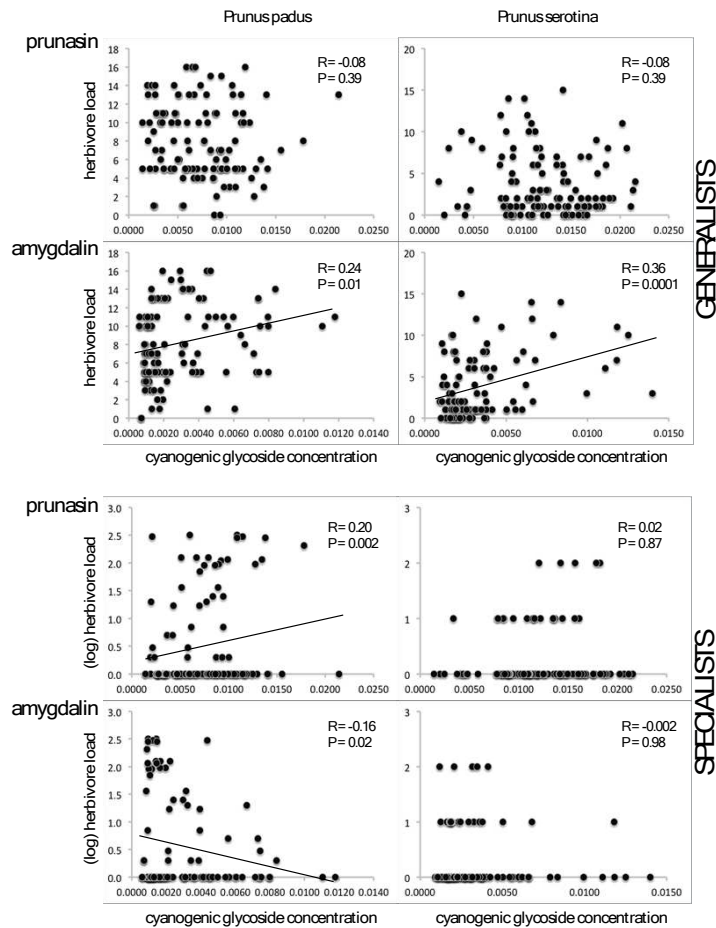
768   herbivores sampled from *Prunus padus* and *Prunus serotina*.

769
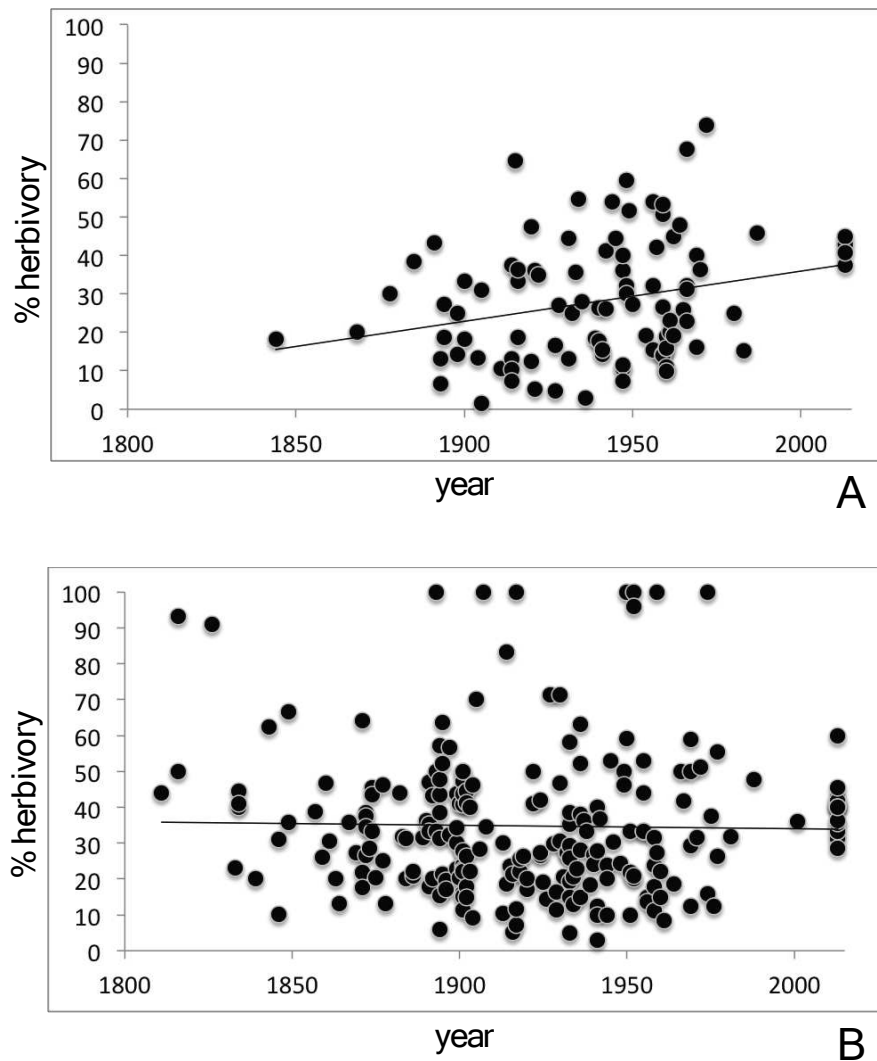
Figure 3. Herbivory over time as derived from herbarium records; A, *Prunus serotina*; B,

Prunus padus.

772

Figure 4. Cyanogenic glycosides and herbivory. *Prunus padus* is shown in the left column,

Prunus serotina in the right column. Data for generalist herbivores are shown in the top

four graphs (separately for prunasin and amygdalin), and for specialist herbivores in the

bottom four graphs (also separately for prunasin and amygdalin). Pearson correlation

coefficients (for the data for generalists) and Spearman's rho (for the data for specialists)

and corresponding *P*-values are given, and regression lines are shown for significant

relationships. Note that the *P*-value for amygdalin vs. specialists in *P. padus* does not

remain significant after Bonferroni correction. Herbivore loads (on the y-axis) are given as

781   counts of individuals per tree, except in the case of specialists on *P. padus*, where the log

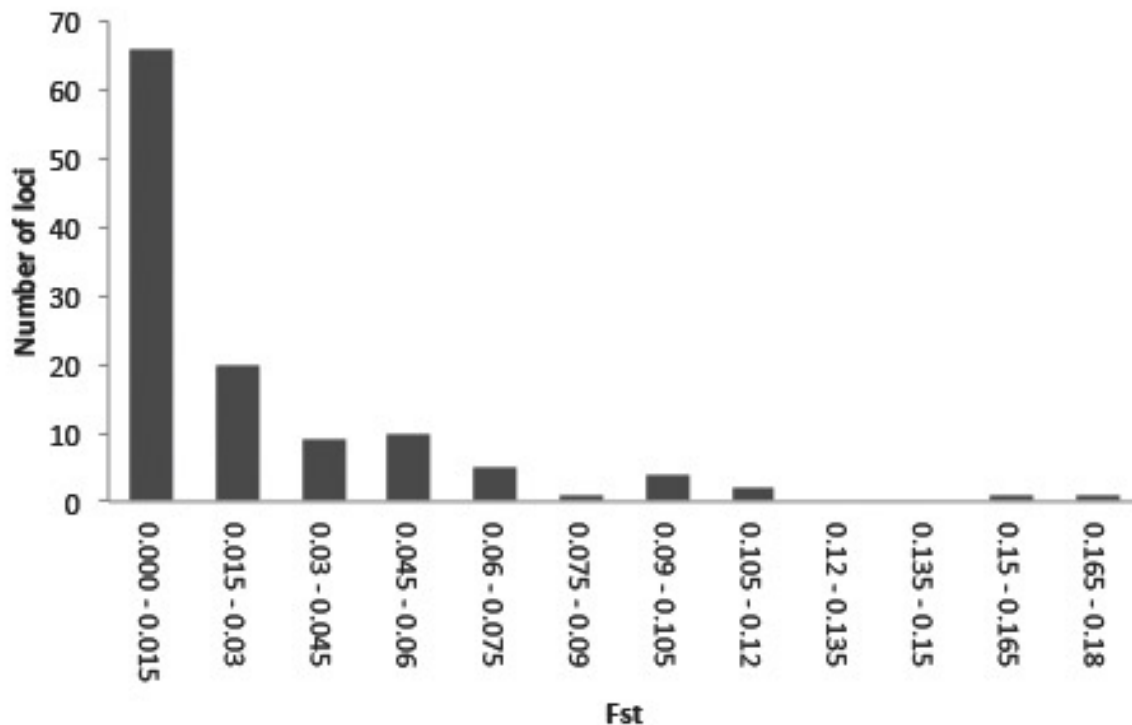782   was taken. Cyanogenic glycoside amounts (on the x-axis) are given as NMR signal integrals.

783

784

785   Figure 5. Frequency distribution of per-locus pairwise (*Prunus-Sorbus*) F_{ST} values for

786   *Gonioctena quinquepunctata*.

787 **Supporting Information (uploaded separately)**

788

789 Table S1. Full data on identities and numbers of herbivores collected on each individual

790 *Prunus serotina* and *Prunus padus* in National Park Zuid-Kennemerland.

791

792 Table S2. Information on insect herbivore damage in historical collection specimens from

793 the National Herbarium, Leiden, for *Prunus serotina* and *Prunus padus.*

794

795 Table S3. Full data on NMR analysis of *Prunus* leaves.

796

797 Table S4. SNP genotype data on *Gonioctena quinquepunctata*.

798

799 Table S5. Oligonucleotides used for the SNP-analysis of *Gonioctena quinquepunctata*.

800

801 Table S6. SNP Loci that showed indications of genetic differentiation between both host

802 plants in the leaf beetle *Gonioctena quinquepunctata*.

803

804 Table S7. ANOVA and GLM results for the *Gonioctena quinquepunctata* host preference

805 tests.

806

807 Table S8. Explanatory variables and response variables for the test of parasitzation of

808 caterpillars on *P. serotina* and *P. padus.*

809

810   SI Text S1. Structure analysis and AMOVA on SNP data for *Gonioctena quinquepunctata*.

811

812   SI Text S2. Calculations of conversion of cyanogenic glycoside contents for dry and fresh

813   weight leaves.