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1 **Title:**

2 **Microevolution of *Pieris* butterfly genes involved in host-plant**
3 **adaptation along a host-plant community cline**

4

5 **Short running title: Microevolution of host-plant adaptation genes**

6

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23

24 **Abstract**

25 Herbivorous insects have evolved counteradaptations to overcome the chemical defenses
26 of their host plants. Several of these counteradaptations have been elucidated at the
27 molecular level, in particular for insects specialized on cruciferous host plants. While the
28 importance of these counteradaptations for host plant colonization is well established,
29 little is known about their microevolutionary dynamics in the field. In this study, we
30 examine patterns of host plant use and insect counteradaptation in three *Pieris* butterfly
31 species across Japan. The larvae of these butterflies express nitrile-specifier protein
32 (NSP) and its paralog major allergen (MA) in their gut to overcome the highly diversified
33 glucosinolate-myrosinase defense system of their cruciferous host plants. *Pieris*
34 *napi* and *Pieris melete* colonize wild Brassicaceae whereas *Pieris rapae* typically uses
35 cultivated *Brassica* as a host, regardless of the local composition of wild crucifers. As
36 expected, NSP and MA diversity was independent of the local composition of wild
37 Brassicaceae in *P. rapae*. In contrast, NSP diversity correlated with local host plant
38 diversity in both species that preferred wild Brassicaceae. *P. melete* and *P. napi* both
39 revealed two distinct major NSP alleles, which shaped diversity among local populations,
40 albeit with different evolutionary trajectories. In comparison, MA showed no indication
41 for local adaptation. Altogether, MA appeared to be evolutionary more conserved than
42 NSP, suggesting that both genes play different roles in diverting host plant chemical
43 defense.

44

45 **Introduction**

46 Herbivorous insects encounter heterogeneous plant communities in the field and often
47 use a subset of those plants as hosts. While feeding, herbivores are exposed to the
48 chemical defenses of their host plants. Defenses typically vary between plant species and
49 populations (Futuyma & Agrawal, 2009; Kliebenstein et al., 2001; Mitchell-Olds &
50 Schmitt, 2006; Prasad et al., 2012; Windsor et al., 2005). Available evidence suggests
51 that specialist herbivores acquired key innovations that enabled them to colonize their
52 host plants by circumventing host plant chemical defenses (Berenbaum, Favret, &
53 Schuler, 1996; Ratzka, Vogel, Kliebenstein, Mitchell-Olds, & Kroymann, 2002; Wheat
54 et al., 2007; Wittstock et al., 2004). The microevolutionary dynamics of these herbivore
55 counteradaptations is largely unknown, despite their ecological and evolutionary
56 importance.

57

58 *Pieris* butterflies use plants from the family Brassicaceae as their hosts. These plants rely
59 on the glucosinolate (GLS)-myrosinase system as their main chemical defense (Wheat et
60 al., 2007; Wittstock et al., 2004; Wittstock & Halkier, 2002). GLSs are hydrolyzed by
61 plant myrosinase enzymes when plant tissue is macerated by insect herbivory, and the
62 main hydrolysis products, isothiocyanates, are highly toxic to most herbivores (Halkier
63 & Gershenzon, 2006; Wittstock & Halkier, 2002). *Pieris* butterfly larvae overcome this
64 defense system by expressing nitrile specifier proteins (NSPs) in their gut. NSPs affect
65 the outcome of GLS breakdown to form nitriles rather than toxic isothiocyanates by an
66 as yet unknown mechanism (Wittstock et al., 2004). Recent genetic and molecular
67 analyses in *Anthocharis cardamines*, another Pierid species feeding on Brassicaceae,
68 indicated that major allergen (MA), an ancient NSP paralog, has NSP-like activity (Edger

69 et al., 2015). Noteworthy, *MA* transcript levels in the gut of *Pieris* larvae are upregulated
70 in response to certain types of GLS (Okamura, Sato, Tsuzuki, Sawada, et al., 2019).
71 Together, *NSP* and *MA* constitute the *NSP*-like gene family, a key innovation that enabled
72 Pierid butterflies to colonize their host plants from the order Brassicales (Edger et al.,
73 2015; Fischer, Wheat, Heckel, & Vogel, 2008; Wittstock et al., 2004).

74

75 GLSs are a highly diverse group of plant secondary metabolites, with about 135 different
76 structures identified in the Brassicales (Blažević et al., 2020). GLS profiles vary both
77 quantitatively and qualitatively (Agerbirk & Olsen, 2012; Fahey, Zalcmann, & Talalay,
78 2001; Olsen et al., 2016) and *Pieris* species typically use several genera within the
79 Brassicaceae as their hosts (Friberg & Wiklund, 2019; Ohsaki & Sato, 1994; Ohsaki,
80 1979; Okamura, Sato, Tsuzuki, Sawada, et al., 2019; Okamura, Sawada, Hirai, &
81 Murakami, 2016). Therefore, each species encounters a range of different GLS profiles
82 in the field, a scenario that should result in local adaptation to host plant communities.
83 Surprisingly, a previous study with *Pieris rapae*, the cabbage white butterfly, found no
84 evidence for local adaptation of *NSP* genes in Europe or in the U.S. (Heidel-Fischer,
85 Vogel, Heckel, & Wheat, 2010). However, this study did not address patterns of host-
86 plant use. Furthermore, *P. rapae*, a considerable pest of *Brassica*, depends mainly on
87 cultivated plants (Grishin et al., 2016; Ryan et al., 2019) and not on local populations of
88 wild Brassicaceae. Hence, it seems important to test species that depend on wild plants in
89 order to detect potential microevolutionary patterns in the genes that underlie insect
90 counteradaptations against their host plants' defenses.

91

92 In this study, we focus on three *Pieris* species, *Pieris melete*, *Pieris napi* and *P. rapae*
93 that co-occur across most of Japan. *P. melete* and *P. napi* use wild Brassicaceae as host
94 plants, including the genera *Arabis*, *Cardamine* and *Rorippa*, but their larvae are rarely
95 observed on cultivated *Brassica* (Ohsaki & Sato, 1994; Okamura, Sato, Tsuzuki, Sawada,
96 et al., 2019). In Japan, plant communities vary substantially along a north-south cline
97 (Kubota, Shiono, & Kusumoto, 2015). Because this holds also true for the latitudinal
98 distribution of wild Brassicaceae, we expected that the plant community cline should
99 affect host-plant use in *P. melete* and *P. napi*, which depend on wild Brassicaceae, but
100 not *P. rapae*, which feeds on cruciferous crops. We sampled local populations of these
101 three species across Japan and acquired data about host plant communities at the sampling
102 sites. We sequenced *NSP* and *MA* and used restriction-site associated DNA sequencing
103 (RAD-seq) data for comparison, to distinguish between the potential impact of selection
104 and neutral genetic processes (Bernatchez & Landry, 2003; Dionne, Miller, Dodson,
105 Caron, & Bernatchez, 2007). As expected, we did not find any evidence for local
106 adaptation of crop-dependent *P. rapae*. In contrast, we found a clear correlation between
107 local host plant diversity and *NSP*, but not *MA*, amino-acid sequence diversity for both
108 *Pieris* species that preferred wild Brassicaceae. Both *P. melete* and *P. napi* possessed two
109 major *NSP* alleles, which caused the observed correlation with host plant diversity, albeit
110 with completely different distribution patterns among Japanese populations. *P. napi*
111 consisted of two distinct populations using very different sets of host plants, with a
112 different *NSP* allele fixed in each population and evidence for directional selection of one
113 allele in the past. In *P. melete*, on the other hand, two *NSP* alleles were maintained by
114 balancing selection across Japan and allele frequency was correlated with local host plant
115 diversity. Altogether, our study indicates that microevolution of counteradaptive traits

116 has an important role for herbivores to adapt to heterogeneous chemical defenses in the

117 host plants that they encounter in the field.

118

119 **Materials and Methods**

120 **Selection of sampling sites**

121 To determine suitable sampling sites, we estimated the diversity of Brassicaceae across
122 Japan with Maxent ver. 3.4.1 (Phillips, Dudík, & Schapire, 2004; Phillips, Anderson,
123 Dudík, Schapire, & Blair, 2017). We used 11,325 individual location data of 44 genera
124 of the Brassicaceae in the Global Biodiversity Information Facility (GBIF.org;
125 <https://doi.org/10.15468/dl.a2nqtv>) and S-Net (<http://science-net.kahaku.go.jp>), and 19
126 climate variables for Japan at 2.5 arc-minute resolution from WorldClim (Fick & Hijmans,
127 2017) to infer additional potential locations of genera from the Brassicaceae (Table S1).
128 We calculated the Shannon index for genera-based diversity of Brassicaceae using the R
129 package “vegan” (Oksanen et al., 2017). Based on this estimation, we chose eleven
130 sampling sites across Japan for each of the three *Pieris* spp., *P. melete*, *P. napi* and *P.*
131 *rapae* (Table S2).

132

133 **Sampling of Brassicaceae and *Pieris* spp.**

134 We collected *Pieris* larvae from April to August 2017. At each sampling site, we used the
135 transect method to search for Brassicaceae along a > 2-km sampling path. We noted the
136 number of plants per species, and collected larvae or eggs of *P. melete*, *P. napi* and *P.*
137 *rapae* from their host plants. We disregarded two Brassicaceae genera, *Capsella* and
138 *Erysimum*, because these are unsuitable hosts for those *Pieris* species (Okamura et al.,
139 2016). Similarly, we did not take into account cultivated Brassicaceae, *i.e.*, cabbage and
140 kale, growing in crop fields near sampling sites. We calculated the host plant diversity
141 using Shannon diversity index for each sampling site, based on the number of species and
142 plants per species. We provided eggs or small larvae with their host plants until they

143 reached the 3rd instar and then dissected the larvae to separate guts from the rest of the
144 bodies. We processed larvae larger than 3rd instar immediately. We stored guts in
145 RNAlater (QIAGEN) and body rests at -80°C. We excluded larvae that contained
146 parasitoid wasps from further analyses.

147

148 **RFLP-based identification of *Pieris* butterfly species**

149 We used restriction fragment length polymorphisms (RFLPs) in the mitochondrial *ND5*
150 gene (GenBank accession numbers: LC090587- LC090590) to determine species identity
151 of larvae. *P. melete* *ND5* has a *HincII* site, and *P. napi* *ND5* has a *HinfI* and a *HindIII* site,
152 whereas *P. rapae* *ND5* lacks these restriction sites. We amplified *ND5* directly from larval
153 bodies, using MightyAmp™ DNA Polymerase Ver.3 (TaKaRa) and *ND5* universal
154 primers (Table S3), followed by restriction and separation on 2% agarose gels. To verify
155 that our species assignments worked correctly, we used the same RFLP technique for
156 adult males previously classified as *P. melete* (N = 24), *P. napi* (N = 16) or *P. rapae* (N
157 = 24) based on differences in the shape of the androconium. *ND5*-based identification
158 matched androconium-based identification for all 64 individuals.

159

160 ***NSP* and *MA* amplification, cloning and sequencing**

161 We randomly picked ten larvae per species and sampling site to sequence *NSP* and *MA*
162 genes. In total, we used 70 larvae (ten larvae per site from seven sampling sites) of *P.*
163 *melete* and *P. napi*, respectively, and 78 larvae of *P. rapae* (ten larvae per site from seven
164 sampling sites, plus eight larvae from Yonaguni Island). We extracted RNA from
165 dissected larval gut using the RNeasy Mini Kit (QIAGEN), synthesized cDNA with the
166 ReverTra Ace qPCR RT Master Mix (TOYOBO) and used TaKaRa Ex Taq (TaKaRa)

167 for amplification of *NSP* and *MA* genes. We gel-purified PCR products with the
168 GEL/PCR Purification Mini Kit (FAVORGEN). We designed PCR primers for *NSP* and
169 *MA* genes based on available RNA-seq data (Accession numbers: ERX2829492-
170 ERX2829499) to confirm that primer-binding sites were conserved and to avoid a
171 potential allelic bias in amplification (Table S3). We cloned it with Mighty TA-cloning
172 kit (TaKaRa), purified plasmids with NucleoSpin Plasmid EasyPure (TaKaRa), and
173 sequenced one plasmid for each *NSP* and *MA* gene from each of the 218 larval samples,
174 using an ABI 3730xl DNA Analyzer (Applied Biosystems).

175

176 **Network analysis of *NSP* and *MA*, and PCR-RFLP of *P. melete* *NSP* gene variants**

177 We trimmed and aligned *NSP* and *MA* reads with MEGA6 (Tamura, Stecher, Peterson,
178 Filipski, & Kumar, 2013) and Mafft (Katoh & Standley, 2013). For each species, we
179 excluded singleton SNPs, assuming that they were PCR errors. We performed
180 NeighborNet network analyses with SplitsTree 4.15.1 (Huson & Bryant, 2006). We used
181 PCR-RFLP to distinguish two major *NSP* variants in *P. melete* to determine whether these
182 variants were different gene copies or alleles. We amplified *NSP* exon 1 from gDNA with
183 EmeraldAmp MAX PCR Master Mix (TaKaRa), using primers specific for exon 1 (Table
184 S3), followed by a *HincII* digest to target a fixed SNP, and separation on 3% TBE agarose
185 gels.

186

187 **RT-qPCR of *P. melete* *NSP* and *MA* genes**

188 To determine whether *NSP* and *MA* gene copy numbers differed among *P. melete*
189 genotypes, we conducted Real Time quantitative PCR (RT-qPCR) from gDNA using
190 *Efla* as a reference gene. For primer design, we used Primer3Plus (Untergasser et al.,

191 2007) with a product size of 70 to 180 bp, a T_m of 59 to 61°C, a GC content of 40 to 60%
192 and a maximum polybase of 3. For RT-qPCR, we used the CFX Connect Real-Time PCR
193 Detection System (Bio-Rad) using TB Green Premix Ex Taq II (Tli RnaseH Plus)
194 (TaKaRa). We used the ddCq method (Pfaffl, 2001) and ANOVA to compare relative
195 gene copy numbers among genotypes.

196

197 **RAD sequencing**

198 We extracted gDNA for RAD-seq from the same 218 individuals that we had used for
199 sequencing of *NSP* and *MA* genes, using the Maxwell 16 LEV Plant DNA Kit (Promega),
200 with *EcoR*I as the restriction enzyme for generating RAD-seq libraries. We ran all 218
201 samples in a single lane on a HiSeq2500 (Illumina SE 50 bp) and trimmed reads with
202 trimmomatic (Bolger, Lohse, & Usadel, 2014), using ILLUMINACLIP:2:10:10,
203 TRAILING:20, SLIDINGWINDOW:4:15, and MINLEN:30. We excluded samples with
204 less than 500,000 reads from further analysis. We called SNPs with stacks ver. 1.48
205 (Catchen, 2013). For ustacks, we set $n = 3$ and $M = 3$, and for cstacks, we used the $n = 3$
206 option. We performed this analysis for each species independently. To acquire values for
207 genetic diversity (π) and genetic distance (F_{st}) of each population, we used “populations”
208 with parameter p set as sampled population number and $r = 0.70$. Since we suspected that
209 *P. melete* and *P. napi* could hybridize, we also called SNPs by setting $p = 1$ and $r = 0.85$
210 in multiple species scales without involving population information for assessing genetic
211 structure of the three species.

212

213 **Population and evolutionary genetic analyses**

214 For analyses of potential population structure we used RAD-seq data in multiple species
215 scales and performed sparse non-negative matrix factorization (SNMF) with the “snmf”
216 function ($K = 1$ to 10, repetition = 20, iterations = 200) implemented in the R package
217 “LEA” (Frichot & François, 2015; Frichot, Mathieu, Trouillon, Bouchard, & François,
218 2014). We used cross-entropy criterion to determine the number of ancestral population
219 (K). We calculated species-wide distributions of Tajima’s D (Tajima, 1989) of *NSP* and
220 *MA* using the poly-div_sfs.pl script (https://github.com/santiagosnchez/poly-div_sfs)
221 iterating random sampling of one individual per population and calculation of D for 300
222 times. We performed these analyses on both, entire coding sequence and individual exons.
223 For calculation of genome-wide Tajima’s D at the species level, we used VCFtools
224 (Danecek et al., 2011). We did the same random sampling coupling with selecting 30
225 random SNPs corresponding to the average number of SNPs in *NSP* within populations.
226 We used poly-div_sfs.pl script to calculate allele frequency of *NSP* and *MA* for each
227 species. At the population level, we used DNAsp ver.6 (Rozas et al., 2017) for calculation
228 of π and F_{st} of *NSP* and *MA*, and Arlequin ver. 3.5 (Excoffier & Lischer, 2010) for
229 Tajima’s D . We estimated F_{st} -like amino acid divergence in *NSP* and *MA* between
230 populations as follows; $F_{st_AA} = \{D_{ij} - \text{mean}(D \text{ within population})\} / D_{ij}$, where D_{ij}
231 shows mean p-distance in amino acid sequences between population i and j . We used the
232 online tool at mkt.uab.es/mkt/MKT.asp (Egea, Casillas, & Barbadilla, 2008) to conduct
233 McDonald–Kreitman tests (McDonald & Kreitman, 1991).

234

235 **Comparison of *NSP* and *MA* with host-plant diversity**

236 We used linear regression to test for each *Pieris* species whether diversity of *NSP* or *MA*
237 correlated with host-plant diversity across sampling sites, using measured host plant

238 diversity as the explanatory variable and NSP or MA amino acid diversity, or genome-
239 wide genetic diversity estimated from RAD-seq data as response variables. Furthermore,
240 we performed a partial Mantel test (Mantel, 1967; Smouse, Long, & Sokal, 1986; Sokal,
241 1979) to test whether NSP or MA amino acid sequence divergence between *Pieris*
242 populations correlated with host-plant community dissimilarities, using the “vegan”
243 package in R (Oksanen et al., 2017). We used the partial Mantel test to compare F_{st} -like
244 amino acid sequence distances of NSP or MA with the Bray-Curtis index for host plant
245 dissimilarity, after controlling for genetic differences between populations (F_{st}) using
246 RAD-seq data. We permuted rows and columns of the first dissimilarity matrix 100,000
247 times to evaluate the reliability of the partial Mantel test results.
248

249 **Results**

250 **1. Field Sampling**

251 **Diversity of Brassicaceae in the field reflects Maxent-based predictions**

252 The Maxent presence-probability estimation predicted that diversity in Brassicaceae
253 communities should be higher in central Japan than in northern and southern regions (Fig.
254 1a). We chose eleven sites along this predicted cline of Brassicaceae diversity: Yubari,
255 Rusutsu and Oshamanbe (all: on the island of Hokkaido), Fukushima, Nagano, Chiba,
256 Kanagawa, Nara (all: on the island of Honshu), Tokushima (on the island of Shikoku),
257 Miyazaki (on the island of Kyushu), and the island Yonaguni (from north to south; Fig.
258 1a). In total, we collected data on 4,777 individual plants across 14 genera and 25 species
259 of the Brassicaceae (Table S2). We used these data to estimate the Shannon index for the
260 diversity of Brassicaceae communities at each site. As expected, field-observed diversity
261 in Brassicaceae communities correlated positively with Maxent predictions ($r = 0.701$, P
262 $= 0.016$), was highest in central Japan, and declined towards the north and the south (Fig.
263 1b).

264

265 **Sampling of *Pieris* larvae in the field**

266 In total, we collected 945 larvae from eleven sampling sites (Table S4). Based on PCR-
267 RFLP using restriction site polymorphisms in the mitochondrial *ND5* region, we
268 identified 483 larvae of *P. melete*, 253 of *P. napi* and 209 of *P. rapae*.

269

270 **2. Population structure of three *Pieris* species in Japan**

271 We randomly chose ten individual larvae per population and species for RAD-seq. On
272 average, we obtained 1,062,550 RAD-seq reads per sample. After removal of samples
273 with low read counts (< 500,000 reads), 183 samples remained for population genetic
274 analyses. Stacks ver.1.48 called 2,490 SNPs for *P. melete*, 1,376 for *P. napi*, and 1,410
275 for *P. rapae*. Genome-wide diversity was higher in *P. rapae* and *P. napi* (both: $\pi = 0.055$)
276 than in *P. melete* ($\pi = 0.042$) (Table 1a). Individual populations of *P. melete* displayed
277 relatively uniform diversity among sampling sites. *P. rapae* populations from central
278 Japan tended to have slightly higher diversity than northern or southern populations.
279 Finally, Hokkaido populations of *P. napi* had higher diversity than other populations in
280 the remainder of Japan (Table 1a)

281 Genome-wide data did not suggest any apparent population genetic structure of *P. melete*
282 or *P. rapae* across Japan, in contrast to *P. napi* (Fig. 2, 3d). In *P. napi*, populations from
283 Hokkaido were distinct from populations in other parts of Japan. In addition, we found
284 evidence for some, very limited gene flow between species (Fig. 2) but not for recent
285 hybridization events among these closely related butterfly species.

286

287 **3. Diversity of *NSP* and *MA***

288 ***NSP* and *MA* sequences are diverse but single copy in *P. melete* and *P. napi***

289 We cloned and sequenced *NSP* and *MA* cDNAs from the same larvae that we used for
290 RAD-seq. PCR products contained near full-length coding sequences (CDS) of *NSP*
291 (1,860 bp of a total CDS of 1,896 bp) and *MA* (1,869 bp of 1,899 bp).

292 Network analysis identified two main clusters of *NSP* variants in both, *P. melete* and
293 *P. napi* (Fig. 3a). In contrast, all *P. rapae* *NSP* sequences grouped together, separate from
294 *P. melete* and *P. napi* *NSPs*. Hokkaido populations of *P. melete* were fixed for one of the

295 main *NSP* variants (Figs. 3a, d), while all other *P. melete* populations possessed both
296 variants, albeit with an overall decline in the frequency of the Hokkaido variant towards
297 the south (Fig. 3d). One of the *P. napi* *NSP* variants was only found in populations from
298 Hokkaido, while the other variant was fixed in central and southern populations (Fig. 3a,
299 d). Network analysis of *MA* showed one single sequence cluster for each of *P. melete* and
300 *P. rapae*, while *P. napi* sequences fell into two distinct clusters (Fig. 3b), with one of the
301 two *P. napi* *MA* variants present only in Yubari on Hokkaido (Figs. 3b, d).

302 To test whether the different *NSP* variants of *P. melete* corresponded to different alleles
303 or two different gene copies, we conducted two experiments, a discrimination of variants
304 with PCR-RFLP and a RT-qPCR-based quantification of gene copy number. The
305 *P. melete* *NSP* variants differed by a SNP causing a *HincII* restriction site polymorphism.
306 After PCR, restriction digest, and agarose gel electrophoresis, we expected to obtain one
307 undigested and one digested PCR product across all individuals if *NSP* variants
308 corresponded to two distinct gene copies. In contrast, we expected to obtain undigested
309 and digested PCR products in various combinations in the case that *NSP* variants
310 corresponded to segregating alleles of the same gene. Indeed, we found samples with only
311 undigested or with only digested PCR products, as well as samples with a mix of both,
312 indicating that *NSP* variants corresponded to different alleles of the same gene (Fig. S1).
313 Furthermore, gene copy numbers determined by RT-qPCR (Fig. S2) from eight randomly
314 chosen samples per genotype were compatible with the same number of *NSP* copies in
315 all *P. melete* genotypes, confirming that *NSP* variants were indeed alleles of the same
316 gene.

317

318 **4. NSP and MA diversity and plant community diversity**

319 **NSP but not MA amino acid diversity correlates positively with host-plant diversity**
320 **in *P. melete* and *P. napi***

321 We found larvae of *P. melete* feeding on eleven, *P. napi* on 14, and *P. rapae* on nine
322 different plant species (Table S4). We found 36% of all *P. melete* larvae on *Rorippa*
323 *indica*, 19% on *Cardamine leucantha*, 18% on *Orychophragmus violaceus*, and 13% on
324 *Arabis hirsuta* (Table S4). *P. napi* larvae were mostly present on *Rorippa sylvestris* (23%),
325 *Arabis flagellosa* (17%), *Cardamine occulta* (17%), *Arabis hirsuta* (12%) and *Barbarea*
326 *orthocera* (11%). Finally, we found *P. rapae* larvae most often on feral plants of *Brassica*
327 *napus* (33%), followed by *Rorippa indica* and *Rorippa sylvestris* (both: 15%), *Cardamine*
328 *kiusiana* (12%), *Nasturtium officinale* and *Rorippa palustris* (both: 10%). However, we
329 also saw a substantial number of *P. rapae* larvae and eggs in crop fields of cabbage,
330 broccoli or kale near sampling sites but we did not have permission to sample from these
331 fields.

332 Altogether, *Pieris* species appeared to use a more diversified set of host plants in central
333 Japan than in northern and southern Japan (Fig. 3c, Table S4). Therefore, we compared
334 diversity in detoxification genes with the diversity of Brassicaceae at sampling sites (Fig.
335 4a). Local host plant diversity correlated significantly with NSP amino acid diversity for
336 both *P. melete* ($r = 0.83$, $P = 0.022$) and *P. napi* ($r = 0.88$, $P = 0.010$), but not *P. rapae*
337 ($r = 0.021$, $P = 0.961$). By contrast, local host plant diversity did not correlate with MA
338 amino acid diversity or genome-wide diversity for any of the three species (Fig. 4b).

339 To confirm the relationship between host plant and NSP sequence, we conducted a partial
340 Mantel test (Mantel, 1967; Smouse et al., 1986; Sokal, 1979). Again, we found positive
341 correlations between host plant dissimilarities and NSP sequence divergence between

342 populations for both, *P. melete* ($r = 0.443$, $P = 0.039$) and *P. napi* ($r = 0.436$, $P = 0.009$)
343 but not *P. rapae*. Likewise, results for MA remained non-significant (Table 2).

344

345 **5. Statistical tests for selection**

346 **Different evolutionary regime between *NSP* and *MA***

347 To identify patterns of potential selection, we calculated Tajima's D for *NSP* (D_{NSP}), *MA*
348 (D_{MA}), and at the genome-wide level (D_{RAD}). D_{RAD} was negative in all three species,
349 compatible with overall population expansion (Fig. 5a). In contrast, D_{NSP} was positive
350 and substantially higher than D_{RAD} in *P. melete* and *P. napi*. Also, D_{MA} was higher than
351 D_{RAD} in *P. rapae*. Furthermore, D_{NSP} varied substantially among exons in *P. melete* and
352 moderately in *P. napi* but not in *P. rapae*, while D_{MA} displayed a more uniform pattern
353 across exons of all three species (Fig. 5b). In *P. melete*, exon 1 had a particularly high
354 positive D . Similarly, *P. napi* exon 3 displayed an elevated D . Close inspection of these
355 exons revealed that both contained several linked, nonsynonymous SNPs at intermediate
356 frequency (Fig. 5c), suggesting that *NSP* was under balancing selection in *P. melete*.

357 In addition, we compared the ratio of non-synonymous to synonymous nucleotide
358 diversity (π_a/π_s) of *NSP* and *MA* of the three *Pieris* spp. *MA* had a lower π_a/π_s ratio
359 compared to *NSP* in all three species (Fig. 5d), suggesting that *MA* was more evolutionary
360 conserved than *NSP* across *Pieris* spp.

361

362 **Divergent selection of *NSP* alleles in *P. napi***

363 We found that *P. melete* and *P. napi* *NSPs*, as well as *P. napi* *MAs*, were all grouped into
364 two clearly distinct clusters (Fig. 3a). Therefore, we tested for potential divergent
365 selection between major alleles, using a population-based diversity test (McDonald &

366 Kreitman, 1991). Results for *P. melete* NSP and *P. napi* MA were non-significant
367 (Table 3), but *P. napi* NSP showed a strong signal of divergent selection (NI = 0.15, $\chi^2 =$
368 10.6, $P = 0.001$), caused by an elevated level of non-synonymous *versus* synonymous
369 substitutions between populations from Hokkaido and populations outside of Hokkaido.
370 In order to identify where mutations had occurred, we compared NSPs of *P. napi* and *P.*
371 *melete*, which allowed us to infer the direction of evolutionary change. To our surprise,
372 all non-synonymous substitutions that differentiated the Hokkaido populations of *P. napi*
373 from those of central and southern Japan had occurred outside of Hokkaido, while each
374 two synonymous substitutions had occurred in the lineages leading to the Hokkaido and
375 non-Hokkaido populations. Furthermore, ten non-synonymous and 24 synonymous
376 polymorphisms were specific for populations from central and southern Japan, and only
377 two non-synonymous polymorphisms were specific for populations from Hokkaido (Fig.
378 6). These patterns of polymorphism indicate that the lineage leading to the extant *P. napi*
379 non-Hokkaido populations experienced strong directional selection on NSP function,
380 followed by stabilizing selection under relaxed constraints, whereas the lineage leading
381 to the extant *P. napi* Hokkaido populations experienced only stabilizing selection.

382

383

384 Discussion

385 Several insect counteradaptations against host plant defenses have been characterized at
386 the molecular level (Berenbaum et al., 1996; Gloss et al., 2014; Heidel-Fischer et al.,
387 2019; Li, Schuler, & Berenbaum, 2003; Ratzka et al., 2002; Wittstock et al., 2004) but
388 only few have been investigated for genetic variation in response to host plant usage.
389 Bono et al. (2008) found some evidence for adaptive evolution of two cytochrome P450
390 genes from the fly *Drosophila mettleri* that were putatively involved in the detoxification
391 of alkaloids from two cactus species. Heidel-Fischer et al. (2010) investigated genetic
392 variation in the *NSP* gene of three European and one US American population of *P. rapae*
393 and found high amounts of amino acid diversity but little evidence for local adaptation.
394 Here, we investigated two genes that underlie the counteradaptation of Pierid butterflies
395 against the GLS-myrosinase defense system of their host plants for potential
396 microevolutionary dynamics in response to host plant usage along a latitudinal cline in
397 Japan. In addition to the crop-dependent *P. rapae*, we included two species that feed on
398 wild Brassicaceae, *P. melete* and *P. napi*. As in the previous study of Heidel-Fischer et
399 al. (2010), we found no evidence for adaptation of *P. rapae* *NSP*-like genes to local
400 communities of wild Brassicaceae. In contrast, both *P. melete* and *P. napi* showed a
401 significant and positive correlation between the diversity of the *NSP* (but not the *MA*)
402 gene product and host plant diversity (Fig. 4). Both species harbored two distinct *NSP*
403 alleles, which shaped the observed correlations in a species-specific manner.
404
405 Hokkaido populations of *P. napi* had a major *NSP* allele that was distinct from alleles
406 found outside of Hokkaido (Fig. 3a). This difference mirrors genome-wide differentiation
407 between both populations (Fig. 2), which suggests two independent colonization events

408 by *P. napi*, one in the north and one in the south of Japan. Differences in the number of
409 acquired polymorphisms in both groups of populations suggest that colonization in the
410 south happened earlier than colonization in the north. Based on the analysis of the
411 mitochondrial *ND5* gene, a recent study concluded that *P. napi* populations on the island
412 of Hokkaido fell into a (south-) western and a (north-) eastern group of populations
413 (Shinkawa et al. 2003), with a hybridization zone in between. Indeed, both Hokkaido
414 populations had elevated genome-wide diversity (Table 1a), and both populations (and in
415 particular Rusutsu) showed signs of gene flow from the south (Fig. 2). Furthermore, *MA*
416 alleles prevalent in the south had apparently replaced the original *MA* alleles in Rusutsu
417 and partially also those in Yubari (Fig. 3d). However, despite gene flow and in contrast
418 to the replacement of *MA* alleles, both Hokkaido populations retained their original *NSP*
419 allele (Fig. 3d). Furthermore, *NSPs* from Hokkaido and from central and southern Japan
420 differed by more non-synonymous substitutions than expected (Table 3), indicative of
421 diversifying selection. However, all amino acid changes that distinguished Hokkaido and
422 non-Hokkaido populations occurred in non-Hokkaido lineage (Fig. 6), indicating that
423 strong directional selection happened in this group, potentially as an adaptive response to
424 novel sets of glucosinolate profiles after colonization. In contrast, both Hokkaido
425 populations displayed very low *NSP* nucleotide diversity, suggesting strong conservation
426 of *NSP* function. This finding is somewhat surprising because *Rorippa sylvestris*, the
427 preferred host plant species of *P. napi* on the island of Hokkaido (Table S4), was
428 introduced only about 50 years ago (Ohsaki, Ohata, Sato, & Rausher, 2020). It would
429 therefore be helpful to characterize the functional properties of *NSP* gene products from
430 both metapopulations to better understand the contrasting patterns of selection on *NSP* of
431 Japanese *P. napi*.

432

433 In contrast to *P. napi*, *P. melete* did not show any indication of population substructure
434 (Fig. 2). In this species, NSP diversity correlated with host plant diversity, which varied
435 along a latitudinal gradient (Fig. 4). Two distinct NSP alleles were present in areas of
436 high host plant diversity, *i.e.*, in central Japan, whereas areas of low host plant diversity
437 were biased towards one of the two alleles, up to fixation of one allele in the Hokkaido
438 populations (Fig. 3d). Although we did not find evidence for diversifying selection,
439 Tajima's *D* was substantially higher for *NSP* than for *MA* or genome-wide sequence, in
440 particular in the first exon (Fig. 5b). This region indeed harbored an elevated number of
441 linked synonymous and non-synonymous SNPs at intermediate frequency (Fig. 5c),
442 suggesting balancing selection acting on this exon across Japan. Balancing selection is an
443 important mechanism to maintain polymorphisms in natural populations, helping
444 organisms to adapt to environments varying in space and time (Hedrick, 2006).
445 Considering the heterogeneous chemical interactions between herbivores and their host
446 plants in the field, it is not surprising to see such polymorphisms maintained in genes
447 involved in these interactions within natural populations. Indeed, there is evidence on the
448 plant side for balancing selection on genes causing variation in chemical defense (Carley
449 et al., 2021; Kroymann, Donnerhacke, Schnabelrauch, & Mitchell-Olds, 2003; Mitchell-
450 Olds & Schmitt, 2006). By contrast, such patterns have previously not been described for
451 counteradaptive traits of herbivorous insects, mainly due to a lack of information about
452 the genetic bases of such traits. Therefore, *NSP* in Japanese *P. melete* may represent the
453 first example for balancing selection acting on an insect counteradaptation against host
454 plant defenses. Of course, functional data would be helpful to substantiate this idea.

455

456 For MA we did not find a clear evolutionary trend. *P. napi* had two distinct MA alleles
457 but these did not show any indication for divergent or balancing selection (Tables 2, 3,
458 Figs. 4, 5). Instead, the comparison of non-synonymous *versus* synonymous diversity
459 suggested that *MAs* were more strongly conserved than *NSPs* in all three *Pieris* species
460 (Fig. 5d). This is consistent with a previous study (Okamura, Sato, Tsuzuki, Murakami,
461 et al., 2019), which found evidence for positive selection only for *NSP* but not for *MA*
462 among different *Pieris* species. Although the functional differences between *NSP* and *MA*
463 are still unknown, both genes displayed distinct expression patterns in response to
464 different GLSs (Okamura, Sato, Tsuzuki, Sawada, et al., 2019). This could indicate that
465 these ancient homologs play different roles in GLS detoxification, with the more
466 evolutionary conserved *MAs* detoxifying more common GLSs, and *NSP* acting on rarer
467 GLSs.

468

469 Remarkably, the same genes show substantial differences in polymorphism patterns and
470 evolutionary trajectories in three different *Pieris* species that populate Japan. This finding
471 can be partially (but not entirely) explained by different host plant usage. The observed
472 different evolutionary dynamics between *NSP* and its paralog *MA* shows that gene
473 duplication and potential functional differentiation appears to be important for adaptation
474 to chemically heterogeneous host plants in the field. Demographic history, and in
475 particular timing and number of colonization events may also play a major role. Therefore,
476 it would be interesting to investigate the same genes, *NSP* and *MA*, in Pierids collected
477 along a latitudinal cline from the eastern coasts of the Asian continent for comparison.

478

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486

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687 **Data Availability Statement**

688 The RAD-seq short read data have been deposited in the EBI short read archive (SRA)
689 with the following project URL: <http://www.ebi.ac.uk/ena/data/view/PRJEB47778>.

690

691 **Author Contributions**

692 Y.O., A.S., L.K., and A.J.N. carried out the laboratory work. Y.O., M.M., H.V., and
693 J.K. conceived, designed and coordinated the study. Y.O., H.V. and J.K. wrote the
694 manuscript. All authors, drafted parts of the manuscript, gave approval for publication
695 and agree to be accountable for the content.

696

697 **Tables and Figures**

698 **Table 1**

699 **(a) Population genetic parameters from genome-wide RAD-seq in three *Pieris***
 700 **species**

Species	Population	private alleles	Individuals	Obs Het	π
<i>P. melete</i>	ALL		57.6	0.0029	0.0042
	Yubari (Hokkaido)	188	8.27	0.0009	0.0012
	Oshamanbe (Hokkaido)	273	9.33	0.0011	0.0013
	Fukushima	181	9.12	0.0010	0.0012
	Nagano	160	8.52	0.0010	0.0012
	Chiba	195	6.20	0.0011	0.0014
	Nara	202	8.81	0.0010	0.0013
	Tokushima	201	8.30	0.0010	0.0013
<i>P. napi</i>	ALL		59.6	0.0034	0.0055
	Yubari (Hokkaido)	269	8.82	0.0013	0.0018
	Rusutsu (Hokkaido)	153	9.18	0.0015	0.0018
	Fukushima	72	8.06	0.0011	0.0015
	Nagano	57	8.94	0.0011	0.0014
	Kanagawa	56	8.59	0.0012	0.0014
	Nara	38	8.61	0.0013	0.0014
	Miyazaki	84	9.39	0.0012	0.0014
<i>P. rapae</i>	ALL		63.7	0.0039	0.0055
	Yubari (Hokkaido)	38	9.55	0.0017	0.0017
	Rusutsu (Hokkaido)	51	8.22	0.0013	0.0017
	Fukushima	36	7.44	0.0013	0.0016
	Nagano	74	8.64	0.0015	0.0019
	Chiba	121	8.19	0.0014	0.0021
	Nara	71	9.45	0.0016	0.0018
	Tokushima	80	8.52	0.0015	0.0019
	Yonaguni	83	6.34	0.0012	0.0015

701

702 (b) Population genetic parameters for *NSP* and *MA* in three *Pieris* species

Species	Sampling sites	Segregating sites (S)		nucleotide diversity (π)		amino acid diversity		Tajima's <i>D</i>	
		<i>NSP</i>	<i>MA</i>	<i>NSP</i>	<i>MA</i>	<i>NSP</i>	<i>MA</i>	<i>NSP</i>	<i>MA</i>
<i>P. melete</i>	ALL	34	44	0.0047	0.0048	0.0070	0.0056	0.5611	-0.2495
	Yubari (Hokkaido)	5	21	0.0007	0.0035	0.0009	0.0032	-1.0353	-0.5370
	Oshamanbe (Hokkaido)	4	23	0.0005	0.0045	0.0003	0.0043	-1.2447	0.1832
	Fukushima	23	20	0.0052	0.0044	0.0080	0.0065	0.7376	0.8698
	Nagano	18	24	0.0047	0.0041	0.0070	0.0049	1.7720	-0.4326
	Chiba	22	16	0.0045	0.0039	0.0065	0.0031	0.4060	0.9582
	Nara	24	31	0.0049	0.0059	0.0069	0.0076	0.3140	0.0867
	Tokushima	27	29	0.0059	0.0052	0.0091	0.0069	0.7735	-0.2318
<i>P. napi</i>	ALL	66	56	0.0098	0.0061	0.0156	0.0087	0.3700	-1.1531
	Yubari (Hokkaido)	5	39	0.0006	0.0056	0.0015	0.0088	-1.3882	-1.2381
	Rusutsu (Hokkaido)	2	11	0.0004	0.0023	0.0012	0.0022	0.1203	0.4869
	Fukushima	27	17	0.0052	0.0029	0.0043	0.0022	-0.1066	-0.3511

	Nagano	29	14	0.0056	0.0021	0.0039	0.0021	0.1320	-0.9809
	Kanagawa	27	15	0.0053	0.0029	0.0041	0.0029	0.1615	0.2393
	Nara	26	14	0.0051	0.0028	0.0043	0.0031	0.1779	0.3970
	Miyazaki	19	10	0.0037	0.0021	0.0012	0.0020	0.1825	0.5833
<i>P. rapae</i>	ALL	43	34	0.0039	0.0038	0.0037	0.0025	-0.7042	-0.0747
	Yubari (Hokkaido)	22	18	0.0029	0.0040	0.0032	0.0033	-1.4854	0.8915
	Rusutsu (Hokkaido)	21	20	0.0033	0.0042	0.0030	0.0020	-1.0311	0.3914
	Fukushima	23	18	0.0047	0.0033	0.0038	0.0024	0.1521	-0.0542
	Nagano	21	15	0.0029	0.0032	0.0028	0.0025	-1.2568	0.6447
	Chiba	18	16	0.0033	0.0038	0.0030	0.0025	-0.6758	1.0538
	Nara	26	14	0.0043	0.0027	0.0038	0.0016	-0.6641	0.2119
	Tokushima	28	22	0.0053	0.0041	0.0052	0.0012	-0.2214	-0.0399
	Yonaguni	18	22	0.0036	0.0046	0.0037	0.0033	-0.4853	0.0754

704 **Table 2 Partial Mantel tests for NSP and MA in three *Pieris* species**

Comparison	Species	<i>r</i>	<i>P</i> -value
Partial Mantel Test			
NSP AA Fst, Plant dissimilarity RAD Fst	<i>P. melete</i>	0.444	0.039
	<i>P. napi</i>	0.436	0.010
	<i>P. rapae</i>	-0.047	0.594
MA AA Fst, Plant dissimilarity RAD Fst	<i>P. melete</i>	0.1405	0.258
	<i>P. napi</i>	-0.318	0.867
	<i>P. rapae</i>	-0.183	0.877

705 NSP AA Fst and MA AA Fst: Fst-like divergences based on amino acid sequences. Plant dissimilarity: Bray-Curtis plant community
 706 dissimilarity. Statistically significant results are in bold ($P \leq 0.05$).

707

708 **Table 3 Population-based tests for diversifying selection (McDonald–Kreitman)**

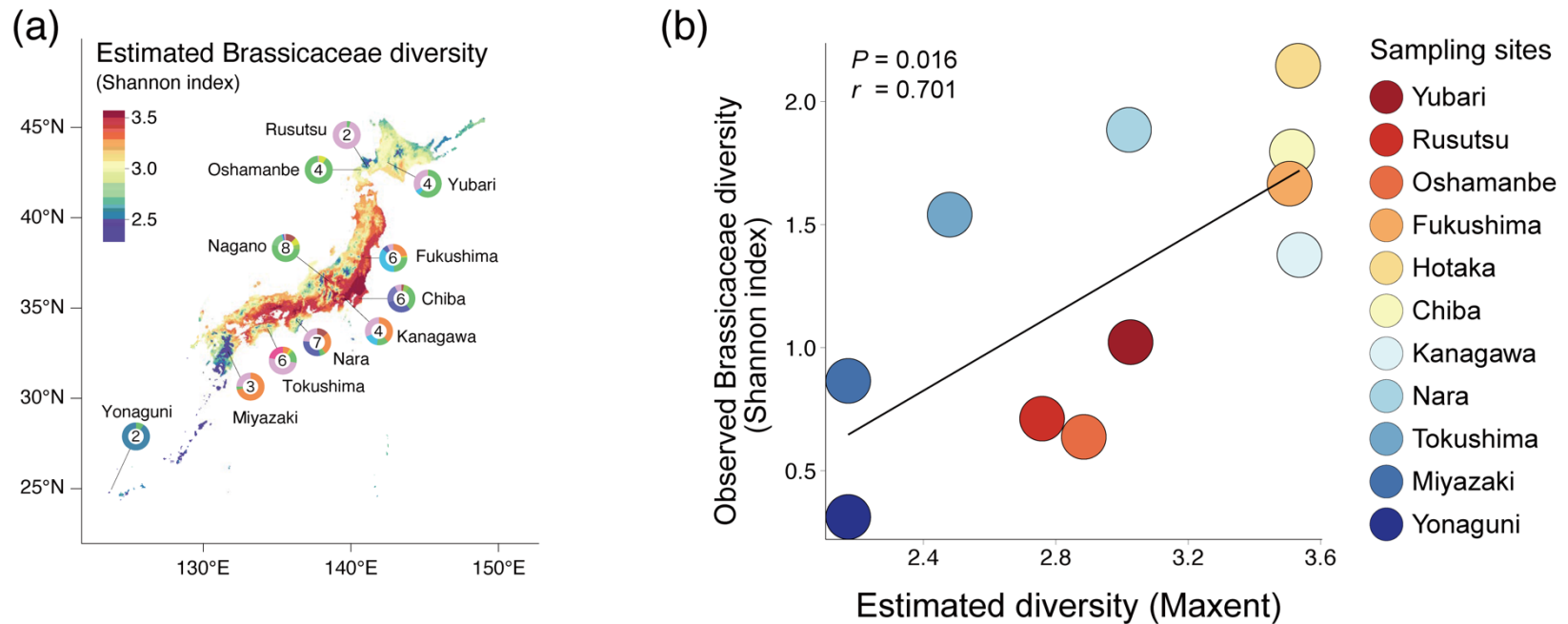
Comparison	Gene	Polymorphisms		Substitutions		NI	X^2	<i>P</i>
		syn	nsyn	syn	nsyn			
<i>P. melete</i>	<i>NSP</i>	20	13	6.05	4	0.982	0	0.981
<i>P. napi</i>	<i>NSP</i>	29	21	4.02	19.16	0.152	10.568	0.001
	<i>MA</i>	22	17	8.1	13.07	0.521	1.807	0.178

709 syn: synonymous, nsyn: nonsynonymous, NI: Neutrality Index

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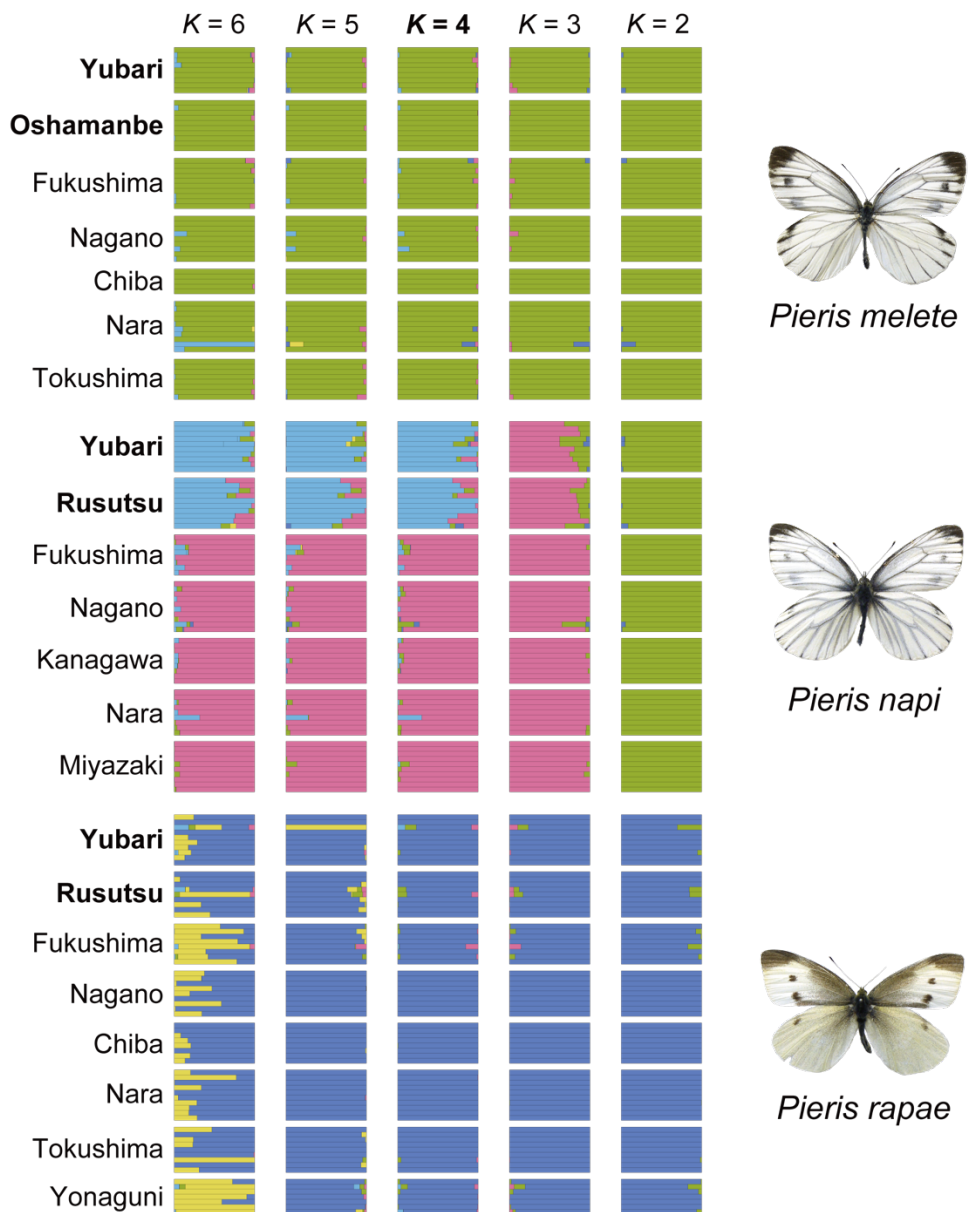


714

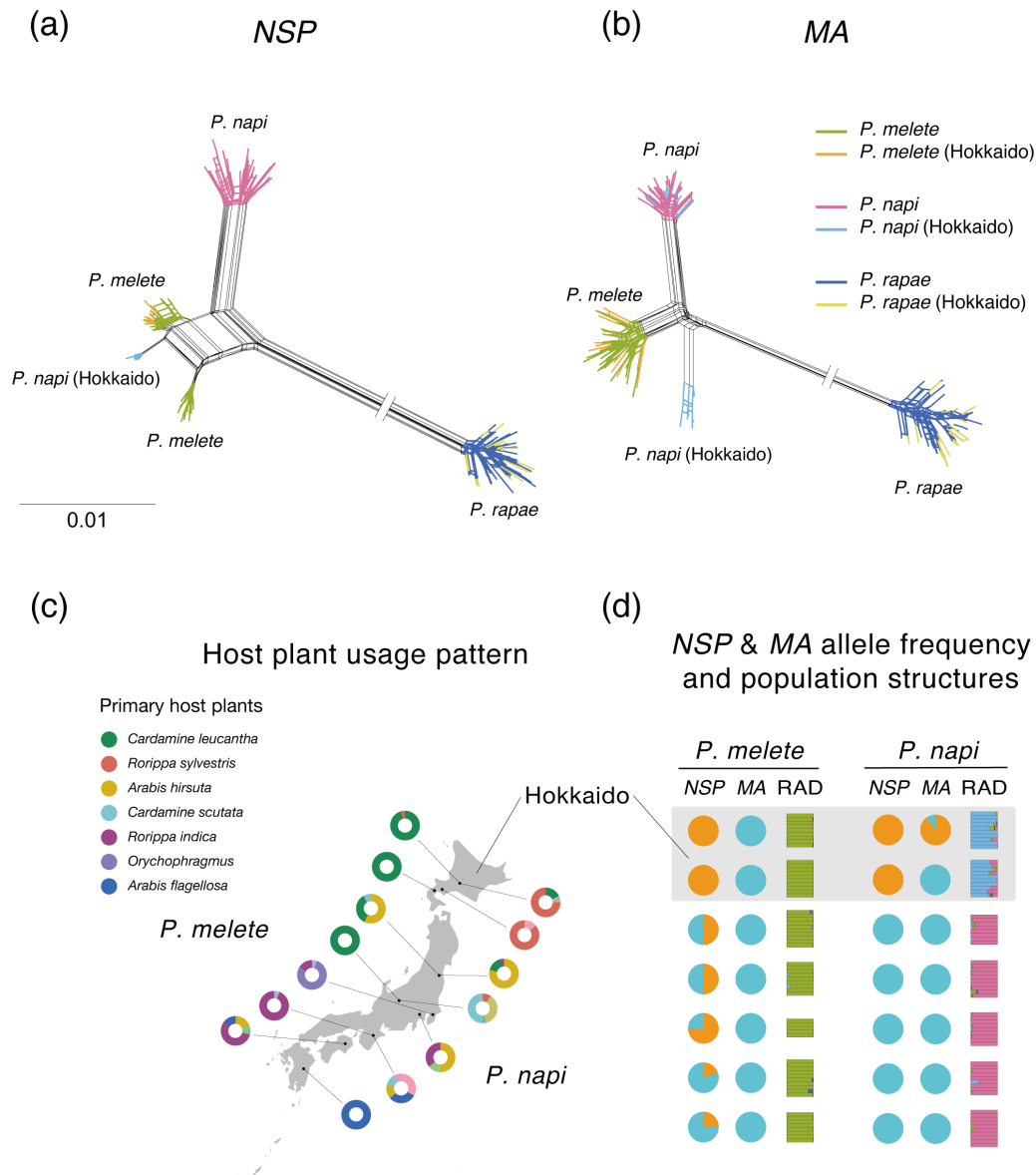
715 **Fig. 1** (a) Maxent-based diversity in Brassicaceae across Japan. The color code indicates the Shannon index for estimated diversity. Pie
 716 charts show number and composition of observed Brassicaceae genera at each sampling site. (b) Pearson correlation between estimated
 717 and observed diversity of Brassicaceae at each sampling site, with the north-south order of sampling sites depicted to the right of the
 718 panel. Diversity in the Brassicaceae is higher in central Japan than in northern or southern Japan.

719

720 **Fig. 2**



729 **Fig. 3**

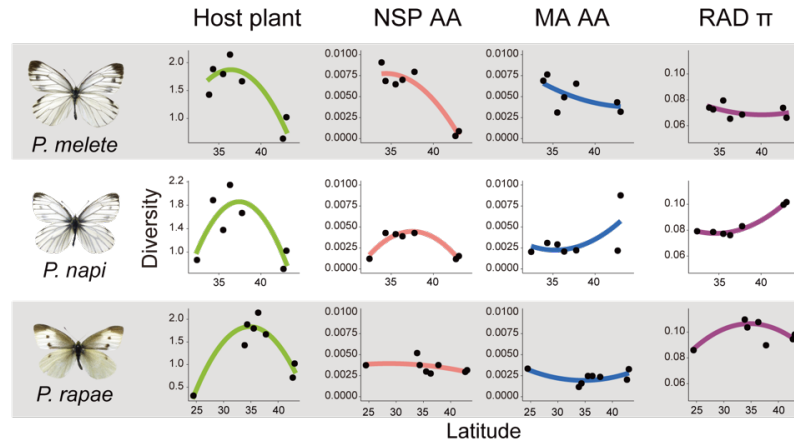


730

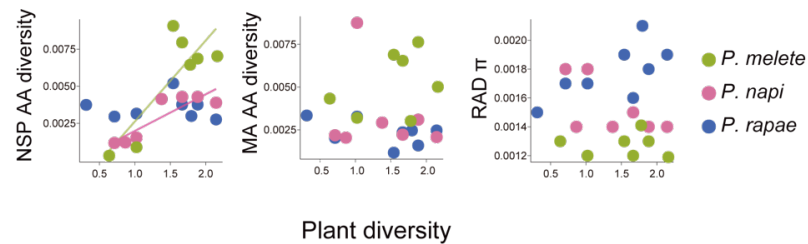
731 **Fig. 3** NeighborNet networks of (a) *NSPs* and (b) *MA*s from three *Pieris* species across
 732 Japan, with different colors for species and for Hokkaido populations. Note that *P.*
 733 *melete* and *P. napi* both have two major *NSP* alleles. Note further that *P. napi* has two
 734 major *MA* alleles. (c) Observed host plant usage patterns of *P. melete* and *P. napi* at
 735 sampling sites. (d) Allele frequencies of major *NSP* and *MA* alleles in comparison with
 736 genome-wide genetic structure of *P. melete* and *P. napi* populations at each sampling
 737 site, ordered from north to south.

738 **Fig. 4**

(a)



(b)



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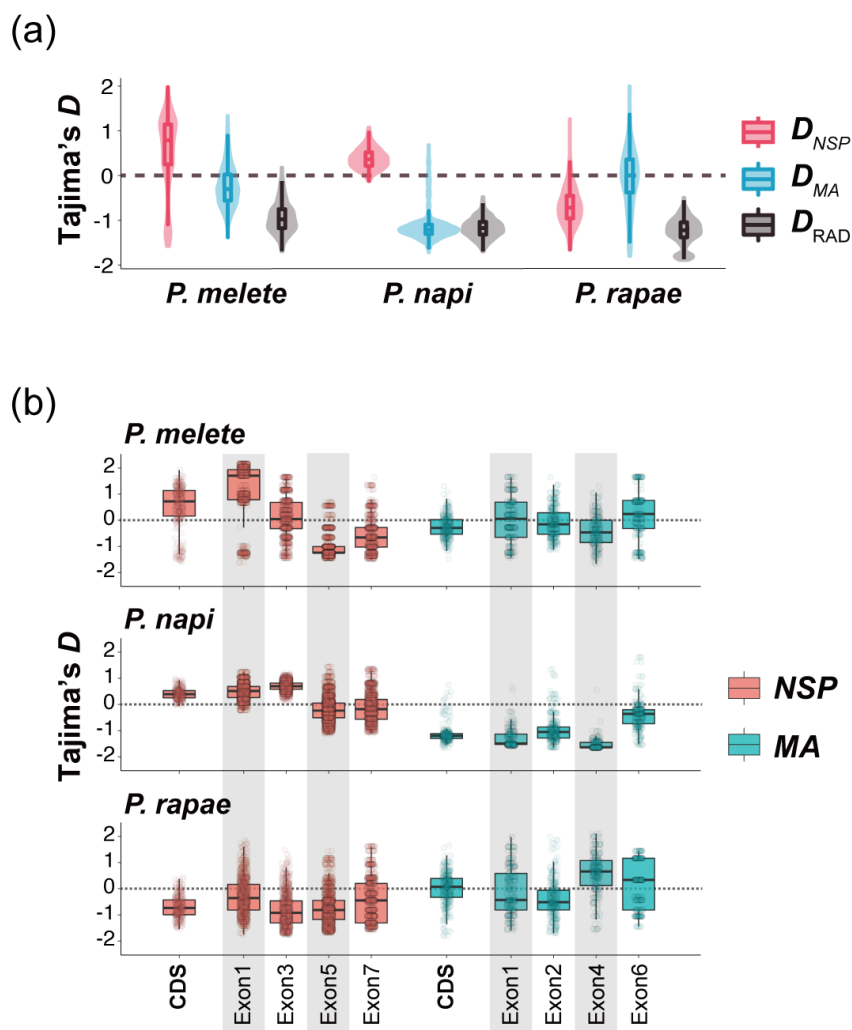
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745

Fig. 4 (a) Host plant diversity (Shannon index), NSP amino acid diversity (NSP AA), MA amino acid diversity (MA AA), and genome-wide diversity (RAD π) within three *Pieris* species at sampling sites ordered according to latitude. Black dots correspond to sampling sites, with colored lines showing regression curves. (b) Correlations between NSP AA, MA AA, or RAD π with host plant diversity in three *Pieris* species. Note the significant positive correlation ($P < 0.05$) between plant diversity and NSP AA diversity in *P. melete* and *P. napi*, but not in *P. rapae*.

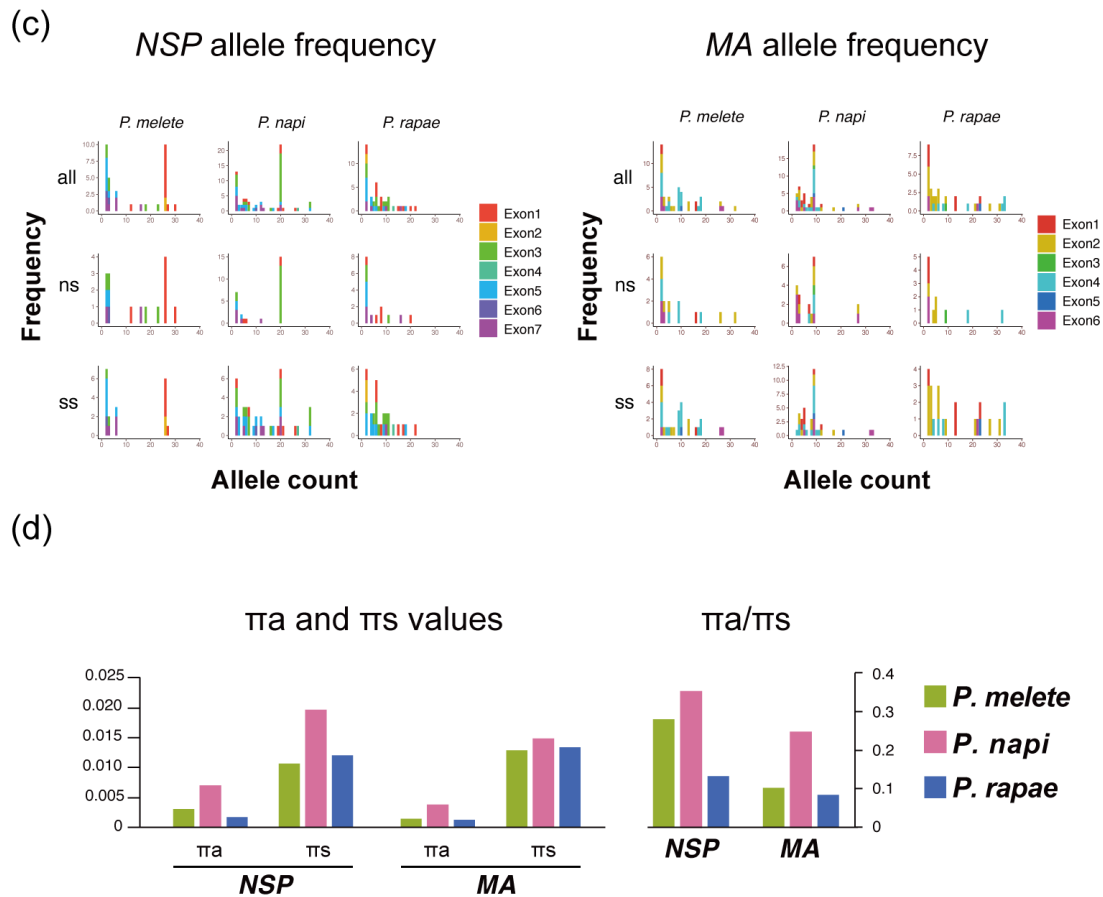
746 Fig. 5



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751 **Fig. 5** (a) Species-wide distributions of Tajima's D for *NSP*, *MA*, and genome-wide data

752 (RAD). Note high values of D for *NSP* in *P. melete* and *P. napi*. (b) Exon-based

753 Tajima's D distribution for *NSP* and *MA*. Only exons > 200 bp are shown. (c) Allele

754 frequency spectra of *NSP* and *MA* based on exon color coding. Exon 1 of *P. melete* and

755 exon 3 of *P. napi* *NSPs* accumulate positions with intermediate allele frequencies. (d)

756 Non-synonymous (π_a) and synonymous nucleotide diversity (π_s) at the species level and

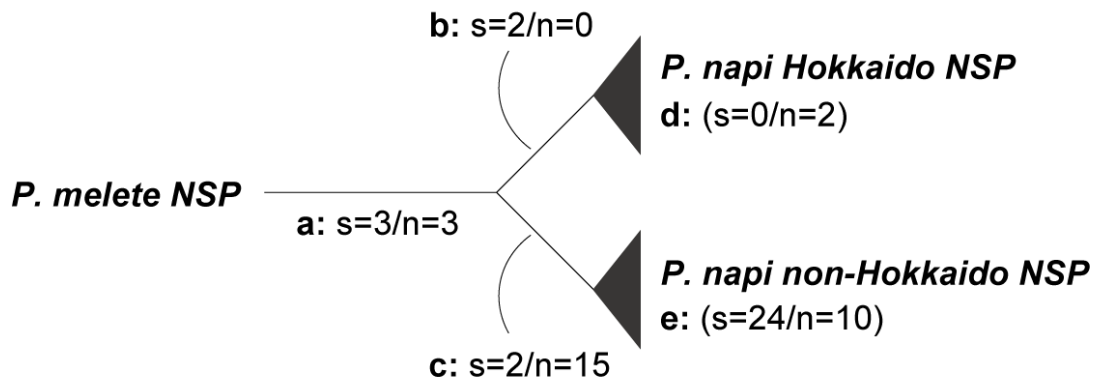
757 ratio of non-synonymous to synonymous diversity (π_a/π_s) of *NSP* and *MA* in three *Pieris*

758 species.

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764 **Fig. 6 Divergent selection on NSP in populations of *Pieris napi*.**

765 Synonymous (s) and non-synonymous (n) mutations in NSPs on branches of a
766 simplified molecular phylogeny consisting of two *Pieris napi* populations and *Pieris*
767 *melete*. Polymorphisms segregating in each population are shown in brackets. A very
768 high number of non-synonymous mutations on the non-Hokkaido branch of *Pieris napi*
769 (branch c) indicates positive selection in the lineage leading to the non-Hokkaido
770 populations of *P. napi*. In comparison, NSP of the Hokkaido populations appears to be
771 highly conserved.

772

773 **Supplementary Tables and Figures**

774 **Table S1 19 climate variables used for Maxent**

Variables	Description
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality (BIO2/BIO7) ($\times 100$)
BIO4	Temperature Seasonality (standard deviation $\times 100$)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

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776

777 **Table S2 Sampling sites and observed number of Brassicaceae**

Sampling Sites	Yubari	Rusutsu	Oshamanbe	Fukushima	Nagano	Chiba	Kanagawa	Nara	Tokushima	Miyazaki	Yonaguni
latitude	43.0	42.7	42.6	37.8	36.3	35.5	35.5	34.3	33.9	32.4	24.5
longitude	142.1	140.8	140.3	140.6	137.8	140.2	139.2	136.0	134.1	131.2	123.0
Plant diversity	1.0224	0.7115	0.6374	1.6662	2.1451	1.7968	1.3765	1.8850	1.5404	0.8650	0.3125
Plant Species											
<i>Arabidopsis thaliana</i>	0	0	0	0	60	20	0	0	0	0	0
<i>Arabidopsis halleri</i>	0	0	0	0	0	0	0	83	0	0	0
<i>Arabidopsis kamchatica</i>	0	0	0	0	46	0	0	0	0	0	0
<i>Arabis flagellosa</i>	0	0	0	0	0	0	0	150	0	245	0
<i>Arabis hirsuta</i>	0	0	0	78	0	0	72	12	12	0	0
<i>Barbarea orthoceras</i>	6	0	15	7	72	0	0	0	4	0	0
<i>Brassica napus</i>	0	0	0	0	51	45	0	10	3	0	0
<i>Cardamine impatiens</i>	0	0	0	0	12	4	7	0	5	0	0
<i>Cardamine kiusiana</i>	0	0	0	0	0	0	0	0	14	0	0
<i>Cardamine leucantha</i>	438	20	130	42	140	0	0	0	0	0	0
<i>Cardamine regeliana</i>	0	0	0	0	81	125	0	0	0	0	0
<i>Cardamine occulta</i>	30	0	12	45	210	60	18	31	0	13	25
<i>Draba nemorosa</i>	0	0	0	0	140	0	0	0	0	0	0
<i>Eutrema japonicum</i>	0	0	0	130	15	0	30	0	0	0	0
<i>Eutrema tenue</i>	47	0	0	0	0	0	0	0	0	0	0
<i>Lepidium virginicum</i>	0	0	0	0	0	0	0	10	0	0	240
<i>Nasturtium officinale</i>	0	0	0	23	0	200	0	174	60	0	0
<i>Orychophragmus violaceus</i>	0	0	0	0	0	140	0	0	0	0	0
<i>Raphanus sativus</i>	0	0	0	0	8	0	0	0	0	0	0
<i>Rorippa dubia</i>	0	0	0	0	0	0	0	62	0	58	0
<i>Rorippa indica</i>	0	0	2	25	0	40	58	75	70	27	0
<i>Rorippa palustris</i>	0	101	0	0	21	10	0	10	0	0	0
<i>Rorippa sylvestris</i>	262	317	0	0	0	0	0	0	0	0	0
<i>Turritis glabra</i>	0	0	0	0	0	0	0	0	30	0	0

779 **Table S3 Primers used in this study**

Primers	Description	Sequence
Primers for cloning and RFLP		
M13_F	M13 Forward	GTTTTCCCAGTCACGAC
M13_R	M13 Reverse	CAGGAAACAGCTATGAC
ND5_F	ND5 Forward	CCTGTTTCTGCTTTAGTTCA
ND5_R	ND5 Reverse	AATATDAGGTATAAATCATAT
NSP_RFLP_F	NSP Forward primer for RFLP (NSPmn_F)	ATGAAAGCTGTTGTAGTCTTATTAGC
NSP_RFLP_R	NSP Reverse primer for RFLP	GTCTTGTACTTCGGACTCCTTTT
Primers for sequencing		
NSPmn_F	NSP Forward for <i>P. melete</i> and <i>P. napi</i>	ATGAAAGCTGTTGTAGTCTTATTAGC
NSPmn_R	NSP Reverse for <i>P. melete</i> and <i>P. napi</i>	CTGTCCGTAAAGAGCAGGTAC
NSPmn_int1	NSP internal1 for <i>P. melete</i> and <i>P. napi</i>	GCTTAGATGCCTTGTC AAAGACT
NSPmn_int2	NSP internal2 for <i>P. melete</i> and <i>P. napi</i>	AATAGCGTGGTCGTTCTTAGC
NSPr_F	NSP Forward for <i>P. rapae</i>	ATGAAAGGTGTTGTAGTCTTCTTAG
NSPr_R	NSP Reverse for <i>P. rapae</i>	TTACTGTCCGTAAAGGGCA
NSPr_int1	NSP internal1 for <i>P. rapae</i>	CTCTGGAAGAACGAAGCATT
NSPr_int2	NSP internal2 for <i>P. rapae</i>	AACTCGGCTAGTCCTGCTTTC-
MAmn_F	MA Forward for <i>P. melete</i> and <i>P. napi</i>	ATGAAGACAACAATAGTGCTCCTAAG
MAr_F	MA Forward for <i>P. rapae</i>	ATGAAGACAACAATAGTGCTCCTC
MAmnr_R	MA Reverse for all the three species	TTATTGTCCCCAGAGGGTTG
MAmn_int1	MA internal1 for <i>P. melete</i> and <i>P. napi</i>	CCTGTTTGAGGAATTACTTCCA
MAmn_int2	MA internal2 for <i>P. melete</i> and <i>P. napi</i>	ATAGGCGTGT TTTGTCCGAAA
MAr_int1	MA internal1 for <i>P. rapae</i>	CTTCCAGCATTCTTCGGAC
MAr_int1	MA internal2 for <i>P. rapae</i>	TCTGATTGCACGATGATGTCC
Primers for RT-qPCR		
NSPm_qPCR_F	Forward qPCR primer for <i>P. melete</i> NSP	AATTGGCGGCTTTATACACG
NSPm_qPCR_R	Reverse qPCR primer for <i>P. melete</i> NSP	TTCTTTCCTTCGGCACTTGT
MAm_qPCR_F	Forward qPCR primer for <i>P. melete</i> MA	TGTTGCTAACGCACTGGAAG
MAm_qPCR_R	Reverse qPCR primer for <i>P. melete</i> MA	CCCTCCAACGCAGTAATGAT
Eflam_qPCR_F	Forward qPCR primer for <i>P. melete</i> Efla	AGGAATTGCGTCGTGGTTAC
Eflam_qPCR_R	Reverse qPCR primer for <i>P. melete</i> Efla	GCAAGCAATGTGAGCTGTGT

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782 **Table S4 Host plant usage patterns of three *Pieris* species at different sampling sites**

783 Columns show ‘observed number of larvae’ and ‘checked number of plants’. Hyphens (-) indicate absence of a plant species at the
 784 sampling site.

<i>P. melete</i>												
Plant sp./Sampling Sites	Yubari	Rusutsu	Oshamanbe	Fukushima	Nagano	Chiba	Kanagawa	Nara	Tokushima	Miyazaki	Yonaguni	SUM
<i>Arabidopsis halleri</i>	-	-	-	-	-	-	-	0/83	-	-	-	0/83
<i>Arabidopsis kamchatica</i>	-	-	-	-	0/46	-	-	-	-	-	-	0/46
<i>Arabidopsis thaliana</i>	-	-	-	-	0/60	0/20	-	-	-	-	-	0/80
<i>Arabis flagellosa</i>	-	-	-	-	-	-	-	0/150	-	0/245	-	0/395
<i>Arabis hirsuta</i>	-	-	-	27/78	-	-	10/72	0/12	28/12	-	-	65/174
<i>Barbarea orthoceras</i>	0/6	-	0/15	0/7	0/72	-	-	-	0/4	-	-	0/104
<i>Brassica napus</i>	-	-	-	-	0/51	0/45	-	0/10	0/3	-	-	0/109
<i>Cardamine impatiens</i>	-	-	-	-	0/12	0/4	9/7	-	6/5	-	-	15/28
<i>Cardamine kiusiana</i>	-	-	-	-	-	-	-	-	10/14	-	-	10/14
<i>Cardamine leucantha</i>	19/438	0/20	24/130	16/42	35/140	-	-	-	-	-	-	94/770
<i>Cardamine occulta</i>	0/30	-	0/12	4/45	0/210	0/60	0/18	2/31	-	0/13	0/25	6/444
<i>Cardamine regeliana</i>	-	-	-	-	0/81	0/125	-	-	-	-	-	0/206
<i>Draba nemorosa</i>	-	-	-	-	0/140	-	-	-	-	-	-	0/140
<i>Eutrema japonicum</i>	-	-	-	0/130	0/15	-	0/30	-	-	-	-	0/175
<i>Eutrema tenue</i>	0/47	-	-	-	-	-	-	-	-	-	-	0/47
<i>Lepidium virginicum</i>	-	-	-	-	-	-	-	0/10	-	-	0/240	0/250
<i>Nasturtium officinale</i>	-	-	-	0/23	-	6/200	-	0/174	0/6	-	-	6/403
<i>Orychophragmus violaceus</i>	-	-	-	-	-	88/140	-	-	-	-	-	88/140
<i>Raphanus sativus</i>	-	-	-	-	0/8	-	-	-	-	-	-	0/8
<i>Rorippa dubia</i>	-	-	-	-	-	-	-	1/62	-	0/58	-	1/120
<i>Rorippa indica</i>	-	-	0/2	0/25	-	18/40	25/58	48/75	85/70	0/27	-	176/297
<i>Rorippa palustris</i>	-	0/101	-	-	0/21	0/10	-	0/10	-	-	-	0/142
<i>Rorippa sylvestris</i>	1/262	0/317	-	-	-	-	-	-	-	-	-	1/579
<i>Turritis glabra</i>	-	-	-	-	-	-	-	-	21/30	-	-	21/30

SUM	20/783	0/438	24/159	47/350	35/856	112/644	44/185	51/617	150/144	0/343	0/265	483/4784
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P. napi

Plant sp./Sampling Sites	Yubari	Rusutsu	Oshamanbe	Fukushima	Nagano	Chiba	Kanagawa	Nara	Tokushima	Miyazaki	Yonaguni	SUM
<i>Arabidopsis halleri</i>	-	-	-	-	-	-	-	11/83	-	-	-	11/83
<i>Arabidopsis kamchatica</i>	-	-	-	-	7/46	-	-	-	-	-	-	7/46
<i>Arabidopsis thaliana</i>	-	-	-	-	0/60	0/20	-	-	-	-	-	0/80
<i>Arabis flagellosa</i>	-	-	-	-	-	-	-	10/150	-	34/245	-	44/395
<i>Arabis hirsuta</i>	-	-	-	19/78	-	-	7/72	5/12	0/12	-	-	31/174
<i>Barbarea orthoceras</i>	0/6	-	0/15	0/7	29/72	-	-	-	0/4	-	-	29/104
<i>Brassica napus</i>	-	-	-	-	0/51	0/45	-	0/10	0/3	-	-	0/109
<i>Cardamine impatiens</i>	-	-	-	-	0/12	0/4	2/7	-	0/5	-	-	2/28
<i>Cardamine kiusiana</i>	-	-	-	-	-	-	-	-	0/14	-	-	0/14
<i>Cardamine leucantha</i>	5/438	0/20	0/130	4/42	0/140	-	-	-	-	-	-	9/770
<i>Cardamine occulta</i>	1/30	-	0/12	0/45	39/210	0/60	0/18	4/31	-	0/13	0/25	44/444
<i>Cardamine regeliana</i>	-	-	-	-	2/81	0/125	-	-	-	-	-	2/206
<i>Draba nemorosa</i>	-	-	-	-	0/140	-	-	-	-	-	-	0/140
<i>Eutrema japonicum</i>	-	-	-	0/130	0/15	-	0/30	-	-	-	-	0/175
<i>Eutrema tenue</i>	1/47	-	-	-	-	-	-	-	-	-	-	1/47
<i>Lepidium virginicum</i>	-	-	-	-	-	-	-	0/10	-	-	0/240	0/250
<i>Nasturtium officinale</i>	-	-	-	0/23	-	0/200	-	0/174	0/6	-	-	0/403
<i>Orychophragmus violaceus</i>	-	-	-	-	-	0/140	-	-	-	-	-	0/140
<i>Raphanus sativus</i>	-	-	-	-	0/8	-	-	-	-	-	-	0/8
<i>Rorippa dubia</i>	-	-	-	-	-	-	-	3/62	-	0/58	-	3/120
<i>Rorippa indica</i>	-	-	0/2	1/25	-	0/40	5/58	0/75	0/70	0/27	-	6/297
<i>Rorippa palustris</i>	-	6/101	-	-	0/21	0/10	-	0/10	-	-	-	6/142
<i>Rorippa sylvestris</i>	21/262	37/317	-	-	-	-	-	-	-	-	-	58/579
<i>Turritis glabra</i>	-	-	-	-	-	-	-	-	0/30	-	-	0/30
SUM	28/783	43/438	0/159	24/350	77/856	0/644	14/185	33/617	0/144	34/343	0/265	253/4784

P. rapae

Plant sp./Sampling Sites	Yubari	Rusutsu	Oshamanbe	Fukushima	Nagano	Chiba	Kanagawa	Nara	Tokushima	Miyazaki	Yonaguni	SUM
<i>Arabidopsis halleri</i>	-	-	-	-	-	-	-	0/83	-	-	-	0/83
<i>Arabidopsis kamchatica</i>	-	-	-	-	0/46	-	-	-	-	-	-	0/46
<i>Arabidopsis thaliana</i>	-	-	-	-	0/60	0/20	-	-	-	-	-	0/80
<i>Arabis flagellosa</i>	-	-	-	-	-	-	-	0/150	-	0/245	-	0/395
<i>Arabis hirsuta</i>	-	-	-	0/78	-	-	0/72	0/12	0/12	-	-	0/174
<i>Barbarea orthoceras</i>	2/6	-	0/15	0/7	0/72	-	-	-	0/4	-	-	2/104
<i>Brassica napus</i>	-	-	-	-	35/51	34/45	-	0/10	0/3	-	-	69/109
<i>Cardamine impatiens</i>	-	-	-	-	0/12	0/4	0/7	-	0/5	-	-	0/28
<i>Cardamine kiusiana</i>	-	-	-	-	-	-	-	-	25/14	-	-	25/14
<i>Cardamine leucantha</i>	0/438	0/20	0/130	0/42	0/140	-	-	-	-	-	-	0/770
<i>Cardamine occulta</i>	0/30	-	0/12	0/45	0/210	0/60	0/18	0/31	-	0/13	0/25	0/444
<i>Cardamine regeliana</i>	-	-	-	-	0/81	0/125	-	-	-	-	-	0/206
<i>Draba nemorosa</i>	-	-	-	-	0/140	-	-	-	-	-	-	0/140
<i>Eutrema japonicum</i>	-	-	-	0/130	0/15	-	0/30	-	-	-	-	0/175
<i>Eutrema tenue</i>	0/47	-	-	-	-	-	-	-	-	-	-	0/47
<i>Lepidium virginicum</i>	-	-	-	-	-	-	-	0/10	-	-	8/240	8/250
<i>Nasturtium officinale</i>	-	-	-	1/23	-	0/200	-	13/174	6/6	-	-	20/403
<i>Orychophragmus violaceus</i>	-	-	-	-	-	0/140	-	-	-	-	-	0/140
<i>Raphanus sativus</i>	-	-	-	-	4/8	-	-	-	-	-	-	4/8
<i>Rorippa dubia</i>	-	-	-	-	-	-	-	0/62	-	0/58	-	0/120
<i>Rorippa indica</i>	-	-	0/2	11/25	-	0/40	0/58	7/75	13/70	0/27	-	31/297
<i>Rorippa palustris</i>	-	20/101	-	-	0/21	0/10	-	0/10	-	-	-	20/142
<i>Rorippa sylvestris</i>	29/262	1/317	-	-	-	-	-	-	-	-	-	30/579
<i>Turritis glabra</i>	-	-	-	-	-	-	-	-	0/30	-	-	0/30
SUM	31/783	21/438	0/159	12/350	39/856	34/644	0/185	20/617	44/144	0/343	8/265	209/4784

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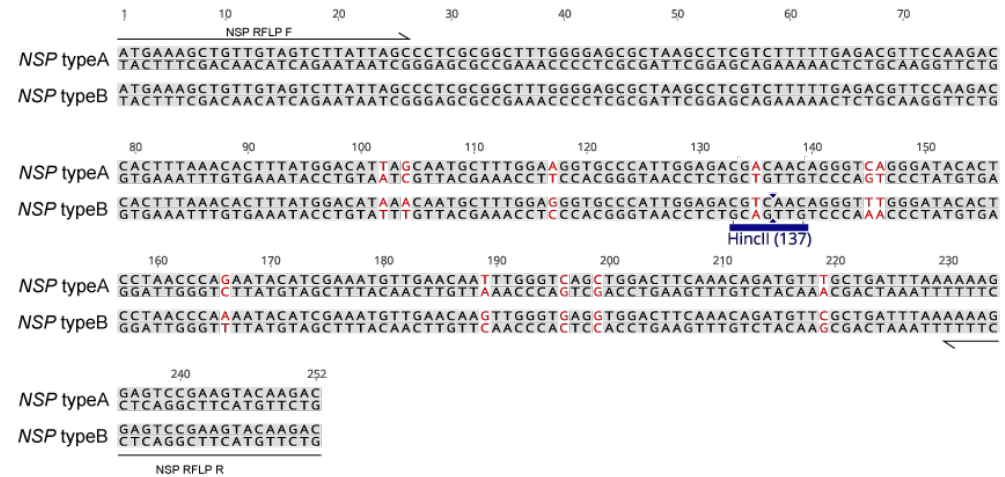
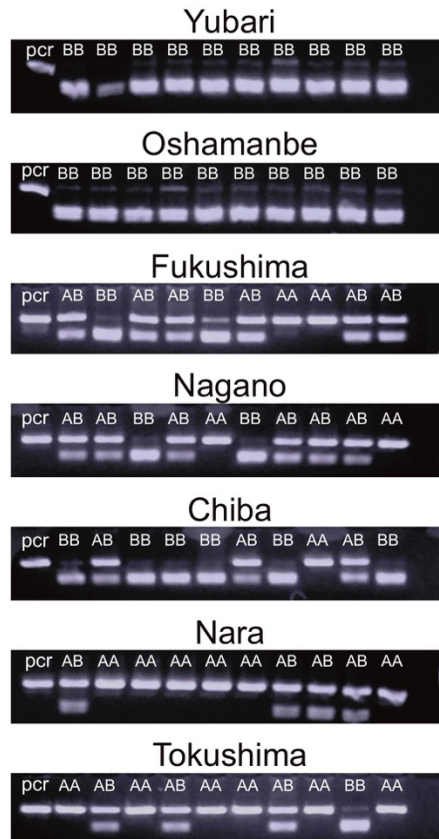
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787 **Table S5 Statistics for *NSP* and *MA***788 (a) *NSP*

Species	Sampling sites	Sequences	Number of sites	S sites	<i>NSP</i> π	AA diversity	Tajima's	<i>P</i> value
<i>P. melete</i>	ALL	69	1860	34	0.0047	0.0070	0.561	-
	Yubari (Hokkaido)	10	1860	5	0.0007	0.0009	-1.035	0.188
	Oshamanbe	10	1860	4	0.0005	0.0003	-1.245	0.116
	Fukushima	9	1860	23	0.0052	0.0080	0.738	0.803
	Nagano	10	1860	18	0.0047	0.0070	1.772	0.980
	Chiba	10	1860	22	0.0045	0.0065	0.406	0.685
	Nara	10	1857-1860	24	0.0049	0.0069	0.314	0.662
	Tokushima	10	1857-1860	27	0.0059	0.0091	0.773	0.815
<i>P. napi</i>	ALL	69	1860	66	0.0098	0.0156	0.370	-
	Yubari (Hokkaido)	10	1860	5	0.0006	0.0015	-1.388	0.094
	Rusutsu (Hokkaido)	10	1860	2	0.0004	0.0012	0.120	0.686
	Fukushima	9	1860	27	0.0052	0.0043	-0.107	0.483
	Nagano	10	1860	29	0.0056	0.0039	0.132	0.584
	Kanagawa	10	1860	27	0.0053	0.0041	0.162	0.610
	Nara	10	1860	26	0.0051	0.0043	0.178	0.614
	Miyazaki	10	1860	19	0.0037	0.0012	0.182	0.615
<i>P. rapae</i>	ALL	74	1869	43	0.0039	0.0037	-0.704	-
	Yubari (Hokkaido)	10	1869	22	0.0029	0.0032	-1.485	0.062
	Rusutsu (Hokkaido)	9	1869	21	0.0033	0.0030	-1.031	0.164
	Fukushima	9	1869	23	0.0047	0.0038	0.152	0.587
	Nagano	10	1869	21	0.0029	0.0028	-1.257	0.105
	Chiba	8	1869	18	0.0033	0.0030	-0.676	0.281
	Nara	10	1869	26	0.0043	0.0038	-0.664	0.265
	Tokushima	10	1869	28	0.0053	0.0052	-0.221	0.518
	Yonaguni (Okinawa)	8	1869	18	0.0036	0.0037	-0.485	0.427

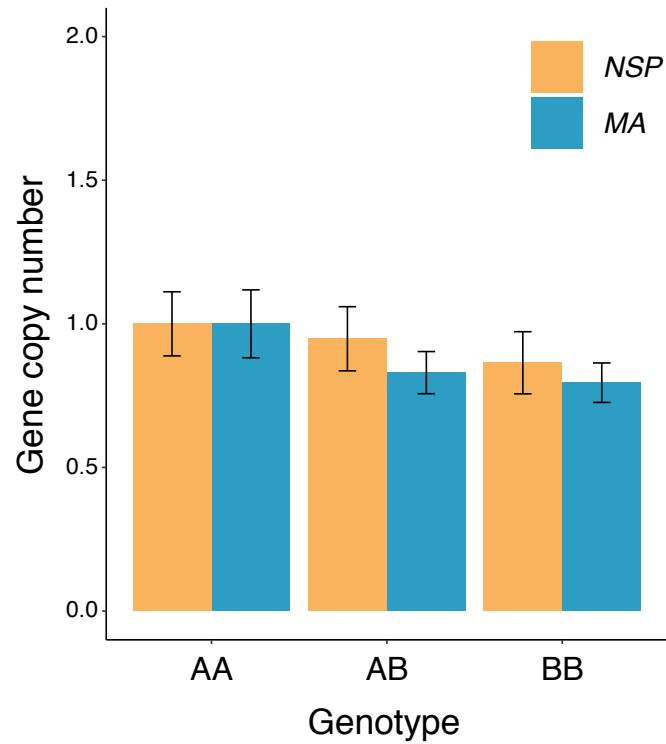
Species	Sampling sites	Sequences	Number of sites	S sites	<i>MA</i> π	AA diversity	Tajima's <i>D</i>	<i>P</i> value
<i>P. melete</i>	ALL	68	1896	44	0.0048	0.0056	-0.2495	-
	Yubari (Hokkaido)	10	1896	21	0.0035	0.0032	-0.5370	0.314
	Oshamanbe	10	1896	23	0.0045	0.0043	0.1832	0.617
	Fukushima	10	1896	20	0.0044	0.0065	0.8698	0.840
	Nagano	10	1896	24	0.0041	0.0049	-0.4326	0.348
	Chiba	8	1896	16	0.0039	0.0031	0.9582	0.859
	Nara	10	1896	31	0.0059	0.0076	0.0867	0.579
	Tokushima	10	1896	29	0.0052	0.0069	-0.2318	0.437
<i>P. napi</i>	ALL	70	1896	56	0.0061	0.0087	-1.1531	-
	Yubari (Hokkaido)	10	1896	39	0.0056	0.0088	-1.2381	0.134
	Rusutsu	10	1896	11	0.0023	0.0022	0.4869	0.711
	Fukushima	10	1896	17	0.0029	0.0022	-0.3511	0.389
	Nagano	10	1896	14	0.0021	0.0021	-0.9809	0.176
	Kanagawa	10	1896	15	0.0029	0.0029	0.2393	0.632
	Nara	10	1896	14	0.0028	0.0031	0.3970	0.687
	Miyazaki	10	1896	10	0.0021	0.0020	0.5833	0.747
<i>P. rapae</i>	ALL	76	1896	34	0.0038	0.0025	-0.0747	-
	Yubari (Hokkaido)	10	1896	18	0.0040	0.0033	0.8915	0.844
	Rusutsu	9	1896	20	0.0042	0.0020	0.3914	0.687
	Fukushima	10	1896	18	0.0033	0.0024	-0.0542	0.508
	Nagano	10	1896	15	0.0032	0.0025	0.6447	0.772
	Chiba	9	1896	16	0.0038	0.0025	1.0538	0.881
	Nara	10	1896	14	0.0027	0.0016	0.2119	0.625
	Tokushima	10	1896	22	0.0041	0.0012	-0.0399	0.513
	Yonaguni	8	1896	22	0.0046	0.0033	0.0754	0.548

790 **Fig. S1(a)** PCR-RFLP of *NSP* exon 1 from seven populations of *P. melete*. ‘pcr’ refers to an undigested PCR product, corresponding to
 791 ‘type A’. Note that ‘type B’ bands in the agarose gels consist of two restriction fragments of similar size. (b) Exon 1 sequences of type A
 792 and type B *NSP* with *HincII* restriction site in type B. SNPs distinguishing types A and B of *NSP* are shown in red.
 793 (a) (b)



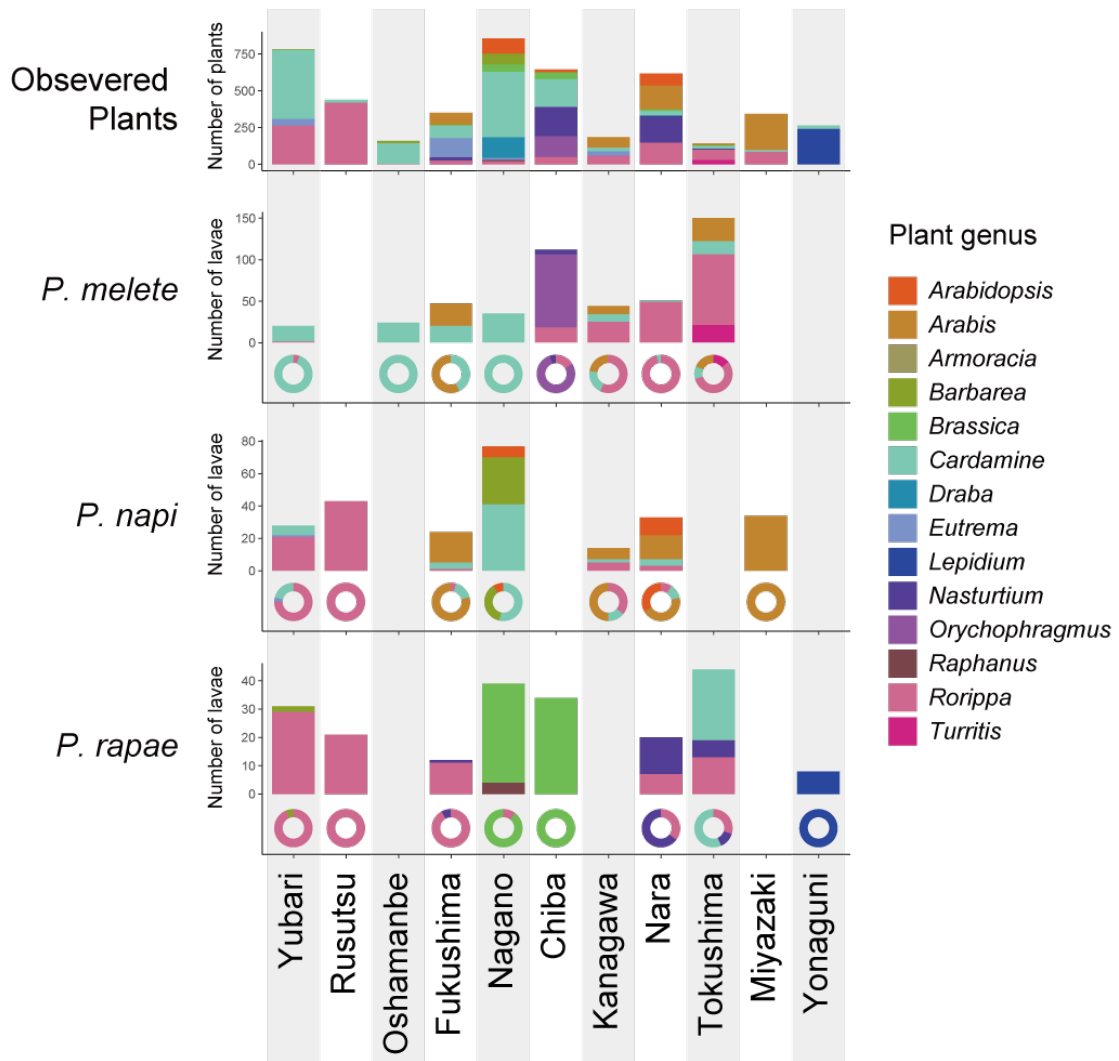
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796 **Fig. S2** *NSP* and *MA* gene copy numbers in *P. melete*. Genotypes (AA, AB and BB) are the same as in Fig. S1. RT-qPCR was done with
797 N = 8 replicates per genotype and *Eflα* as a reference gene. Data were standardized with mean of genotype AA set as 1. Bars shows
798 means (\pm SE). Genotypes do not differ in *NSP* (yellow bars) or *MA* (blue bars) gene copy numbers.



799

800 **Fig. S3** Patterns of host plant use of *Pieris* species in the field. The plant species data
 801 were summarized to genus level for visualization. The pie charts show composition of
 802 plant genus and each bar show number of individuals (either plants or larvae).



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