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Molecular signatures of selection associated with host-plant differences in *Pieris* butterflies

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Abstract

Adaptive traits that enable organisms to conquer novel niches and experience subsequent diversification are ecologically and evolutionarily important. The larvae of *Pieris* butterflies express nitrile-specifier proteins (NSPs), a key innovation for overcoming the glucosinolate (GLS)-myrosinase-based defense system of their Brassicales host-plants. NSPs are a member of the NSP-like gene family, which includes the major allergen (MA) protein, a paralog of NSP with a GLS-disarming function, and a single domain major allergen (SDMA) protein, whose function is unknown. The arms-race between a highly variable host-plant defense system and members of the NSP-like gene family is suggested to mediate diversification in both Pierid butterflies and Brassicales plants. Here, we combined feeding experiments using 25 Brassicaceae plants and five *Pieris* species with larval transcriptome data to investigate the evolutionary forces acting on NSP-like gene family members associated with patterns of host-plant usage. Although we observed significantly elevated nonsynonymous to synonymous substitution ratios in NSPs, no such pattern was observed in MAs or SDMAs. Furthermore, we found a signature of positive selection of NSP at a phylogenetic branch which reflects different host-plant preferences. Our data indicate that NSPs have evolved in response to shifting preferences for host plants among five *Pieris* butterflies, whereas MAs and SDMAs appear to have more conserved functions. Our results show that the evolution and functional differentiation of key genes used in host-plant adaptation play a crucial role in the chemical arms-race between *Pieris* butterflies and their Brassicales host-plants.

Introduction

Key innovations that enable organisms to acquire novel niches and experience subsequent radiation are ecologically and evolutionarily important (Bond & Opell, 1988; Hunter, 1998). In plant-herbivore interactions, a number of key innovations were identified that enabled herbivores to overcome specific plant defense mechanisms and colonize novel host-plants (Berenbaum, Favret, & Schuler, 1996; Janz, 2011; Wheat et al., 2007). *Pieris* butterfly larvae use plants containing glucosinolate (GLSs) as hosts, redirecting toxic breakdown products to less toxic metabolites using gut-expressed nitrile-specifier proteins (NSPs) (Wittstock et al., 2004). NSPs are known to be a key innovation of *Pieris* butterflies: the acquisition of NSPs enabled *Pieri*s to colonize GLS-containing Brassicales followed by higher speciation rates compared to those of sister butterfly clades (Edger et al., 2015; Fischer, Wheat, Heckel, & Vogel, 2008; Heidel-Fischer, Vogel, Heckel, & Wheat, 2010; Wheat et al., 2007).

NSPs are members of the small NSP-like gene family, which includes major allergen (MA) proteins and single domain major allergen (SDMA) proteins (Fischer et al., 2008). The functions of MAs and SDMAs are mostly unclear, however, the structures of MAs and NSPs are known to be similar: three replicated domains originated from SDMAs (Fischer et al., 2008). In addition, although SDMAs are generally expressed in the guts of Lepidopteran larvae (Randall, Perera, London, & Mueller, 2013), NSP and MA are only found in Pierid butterflies feeding on Brassicales (Fischer et al., 2008). These findings suggest that in *Pieris*, MAs, as in NSPs, have a function related to disarming GLSs. The ability of MAs to redirect GLS hydrolysis was recently documented in one Brassicales-feeding Pierid, *Anthocharis cardamines*, which seems to have MA genes only, that is, it lacks NSPs (Edger et al., 2015). Thus, although the function of MAs in

Pieridae is largely unknown, especially in those species which have NSPs and MAs, MAs also appear to be ecologically important for overcoming the host plant's GLS-based defense system.

Previous studies indicated that the co-evolutionary diversification of Brassicales plants and Pierid butterflies was mediated by the chemical arms-race between the glucosinolate-myrosinase defense system and members of the NSP-like gene family (Edger et al., 2015). Past increases of GLS complexity in Brassicales were followed by the evolution in Pierid butterflies of NSP-like gene family members, suggesting that members of the NSP-like gene family would potentially be under strong selection pressure, were Pieridae butterflies to expand or shift their host plants. Such a scenario is supported by recent findings of signatures of positive selection in partial NSP sequences of a pair of *Pieris* butterflies in comparison with the signatures of 70 randomly selected genes (Heidel-Fischer et al., 2010). However, the evolutionary forces acting on all NSP-like gene family members, especially when considering the associated host plant spectrum, remains unknown.

Here, we focus on five Japanese butterfly species (*Pieris napi*, *P. melete*, *P. rapae*, *P. brassicae* and *P. canidia*) in the genus *Pieris*, which has both NSP and MA genes, and feed on Brassicaceae plants with the highest GLS diversity among the Brassicales. The five *Pieris* species have different host spectra according to field observations (Fig. 1), with *P. napi* and *P. melete* frequently using wild Brassicaceae plants (such as *Arabis* or *Arabidopsis*), whereas *P. rapae* and *P. brassicae* tend to feed on Brassicaceae crops and are known as major pests (Benson, Pasquale, Van Driesche, & Elkinton, 2003; Kitahara, 2016; Ohsaki & Sato, 1994; Ueno, 1997). In contrast, in Japan, *P. canidia* can be found only in the southern islands (Yonaguni Island, Okinawa), relying on the limited number of host plants, such as *Cardamine* or *Lepidium,* in their habitat range.

With larval transcriptome (RNA-seq) data from the five *Pieris* species, we analyzed the

85 divergence in amino acid sequences based on nonsynonymous (dN) and synonymous substitution
86 (dS) rates to investigate signatures of selection on members of the NSP-like gene family compared
87 with signatures on other larval-expressed orthologs. We also conducted comprehensive feeding
88 experiments with 25 Brassicaceae plants to acquire patterns of host utilization in *Pieris* species to
89 test if shifts in these patterns can be correlated with the evolution of NSP-like gene family members
90 in *Pieris* butterflies. Additionally, we searched for functional gene groups with signatures of
91 selection among the five *Pieris* species based on gene ontology (GO) and dN/dS analyses to
92 identify potential genes related to host-plant detoxification which might be under positive or
93 negative selection. By combining these approaches, we were able to investigate signatures of
94 selection on ecologically important NSP-like gene family members and detoxification-related
95 genes associated with host-plant utilization patterns in *Pieris* larvae (Fig. 2). Results provide
96 important insights into the evolution of adaptive key innovations in *Pieris* butterflies.
97
98

99 # Materials and Methods

100 ## Feeding experiments

101 We used four *Pieris* butterfly species for the feeding assay, leaving out *P. canidia*. We collected 7–
102 10 female butterflies of three *Pieris* butterfly species (*P. napi*, *P. melete*, *P. rapae*) from wild
103 populations in Chiba and Hokkaido, Japan. Most wild-caught female butterflies were already
104 fertilized. We released the female butterflies into cages containing cabbage (*Brassica oleracea* var.
105 *capitata*) or *Cardamine leucantha* under high-intensity light conditions, and waited for eggs to be
106 laid. For *P. brassicae*, final-instar larvae were caught in the wild (Hokkaido, Japan), fed on cabbage
107 and reared to the adult stage. After eclosion, 10 female butterflies were hand paired with males and
108 eggs were collected as they were from the other species. Eggs of the four *Pieris* butterfly species
109 were incubated at 25°C until they hatched.

110 For experimental plants, we collected seeds of 25 Brassicaceae plant species, covering a
111 phylogenetically broad range (Table S1) (Beilstein, Al-Shehbaz, Mathews, & Kellogg, 2008;
112 Couvreur et al., 2010; Franzke, Lysak, Al-Shehbaz, Koch, & Mummenhoff, 2011). We grew the
113 plants in the greenhouse at 25°C, with 60% relative humidity and L16:D8. Plants were watered and
114 fertilized every week with a 2000× diluted solution of Hyponex (N:P:K = 6:10:5; Hyponex, Osaka,
115 Japan). After two months of cultivation, plants were used for the feeding experiments.

116 Neonate larvae were collected within 12 hours after they hatched for the feeding
117 experiment. We transferred three neonate larvae for each plant using a soft-haired brush and
118 replicated this twice for each plant species (n = 6). To minimize changes in the condition of the
119 experimental plants, experimental trials were carried out within 5 days for all four *Pieris* species.
120 We conducted feeding experiments under the same temperature and light conditions used for plant

121 growth. We measured the weight of each larva individually (within 0.1 mg) after 120 hours of
122 feeding and used the average weight of larval individuals from each plant species as an index of
123 the performance of each *Pieris* butterfly species. We set the weight of dead larvae at 0.
124 Larval weights were standardized as z-scores to enable comparison between species. We
125 calculated the mean scores of each plant treatment and used these for the comparative analysis. We
126 conducted Pearson's correlation test and hierarchical clustering analysis to assess differences in
127 larval performances among the four *Pieris* species. The possible clustering was evaluated with the
128 gap statistics (Tibshirani, Walther, & Hastie, 2001). All of these analyses were performed on R
129 studio ver. 1.1.453 (RStudioTeam, 2016).
130
131 **RNA sequencing**
132 From four *Pieris* butterfly species (*P. napi*, *P. melete*, *P. rapae, P. brassicae*), excluding *P. canidia,*
133 we collected larvae that we used for the feeding experiments for transcriptome analysis (Figs. 1,
134 2). We used larvae that fed on *Arabidopsis kamchatica* and *Cardamine occulta* as representatives.
135 The larvae were flash-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. We
136 selected a single representative larva for each of the four *Pieris* and plant species combinations,
137 and RNA was extracted using the RNeasy Mini Kit (QIAGEN). RNA sample quantity and quality
138 were checked by Agilent 2100 Bioanalyzer. Illumina libraries of individual larva were prepared by
139 Sure Select Strand-Specific RNA Library Preparation Kit for Illumina Multiplexed Sequencing,
140 and RNA sequencing was performed on an Illumina HiSeq 1500 Genome Analyzer platform using
141 a 2 x 100bp paired-end approach. For *P. canidia*, we collected larvae directly from wild *Lepidium*
142 *virginicum* on Yonaguni Island, Okinawa, Japan. The collected larvae were dissected, and gut

143 tissues were stored at -80 °C in solution until RNA extraction. Five larvae were randomly selected,
144 and RNA was extracted with the RNeasy Mini Kit (QIAGEN). *P. canidia* RNA concentrations were
145 quantified on a Qubit 2 Fluorometer (Invitrogen), and a fraction of the RNA from each of the five
146 larvae was pooled as a single sample for RNA-seq. Paired-end (2×150 bp) sequencing was
147 performed by the Max Planck Genome Center Cologne on an Illumina HiSeq 2500 Genome
148 Analyzer platform.
149
150 ***De novo* assembly, searching for reciprocal best hits (RBHs) using-BLAST**
151 Acquired reads of RNA-seq data were pooled for each species after filtering out bad quality reads
152 by trimmomatic with the following options (LEADING:10 TRAILING:10
153 SLIDINGWINDOW:4:20 MINLEN:40) (Bolger, Lohse, & Usadel, 2014). The quality of reads was
154 checked by FastQC. Pooled reads were *de novo* assembled by Trinity ver. 2.0.6 (Grabherr et al.,
155 2011). We used TransDecoder (http://transdecoder.github.io/) to predict open reading frames
156 (ORFs) from the assembled contigs and subsequently looked for reciprocal best hits (RBHs) using
157 BLAST alignment methods to analyze amino acid sequences (longer than 100 amino acids)
158 predicted by TransDecoder (Camacho et al., 2009; Cock, Chilton, Grüning, Johnson, & Soranzo,
159 2015). We used RBH BLAST software with default settings on all possible species pairs (10 pairs)
160 and subsequently extracted *P. rapae* orthologs from this RBH result and ran blastp on the amino
161 acid sequences against a *P. rapae* protein database to confirm the ORF prediction from
162 TransDecoder. Orthologs in the RBH result without any BLAST hits to the *P. rapae* protein
163 database were removed since these amino acid sequences may have resulted from wrong ORF
164 predictions by TransDecoder. We used PRANK to conduct codon-based alignment of each ortholog
165 set acquired from the RBH result (Loytynoja & Goldman, 2005).

166

167 **Phylogenetic tree construction**

168 We reconstructed a phylogeny of the five *Pieris* species using the transcriptome data by
169 concatenating all aligned ortholog nucleotide sequences into one sequence for each species,
170 generating an Maximum Likelihood (ML) phylogenetic tree by IQ-tree (Nguyen, Schmidt, Von
171 Haeseler, & Minh, 2015) after removing gaps with TrimAl (2063074 bp remaining) (Capella-
172 Gutiérrez, Silla-Martínez, & Gabaldón, 2009). We used the GTR + gamma substitution model and
173 set ultrafast bootstrap approximation iterations as 1000, using -bnni options to construct a
174 phylogeny of the five *Pieris* species (Hoang, Chernomor, Von Haeseler, Minh, & Vinh, 2018).

175

176 **Analysis of NSP-like gene family members**

177 We used each aligned orthologous gene for calculating species pairwise dN/dS ratios using PAML
178 4.8 (Yang, 2007). We used runmode = -2 and NSsites = 0 option in codeml from PAML and
179 calculated pairwise dN/dS ratios based on the Nei & Gojobori method (Nei & Gojobori, 1986). We
180 averaged acquired species pairwise dN/dS ratios for each ortholog to infer putative positive
181 selection among the genus. The dN/dS values of NSP-like gene family members were compared
182 with the entire dN/dS distributions of all ortholog sets in species pairwise associations and also in
183 an averaged dN/dS scale among *Pieris*.

184 We used the branch-site model to identify cases of positive selection on NSP-like gene
185 family members in a specific branch. We prepared molecular phylogeny of NSP gene family
186 members by RAxML (Stamatakis, 2014) and tested all branches using codeml model 2 with
187 NSsites = 2 option and ran an alternative model; varied dN/dS ratios across sites as well as lineages
188 were allowed (fixed_omega = 0), and null model; fixed dN/dS (fixed_omega = 1). We conducted

189 a likelihood ratio test (LRT) with the chi-square distribution to evaluate significant differences
190 between the alternative and null models. We also used adaptive Branch-Site Random Effects
191 Likelihood (aBSREL) analysis for the NSP-like gene members among the five *Pieris* species and
192 tested all branches to identify the signatures of positive selection (Smith et al., 2015). Acquired *P*
193 values were corrected with false discovery rates (FDRs) in each analysis. Signs of positive selection
194 on each site were identified by the Bayes empirical Bayes (BEB) analysis (0.90 cut offs). The
195 aBSREL analyses were performed in HyPhy implemented in the datamonkey web server
196 (Kosakovsky Pond, Frost, & Muse, 2005; Weaver et al., 2018).
197
198 **GO annotation and evolutionary tests**
199 We used *P. rapae* contigs from the RBH result for GO annotation and ran these genes against the
200 NCBI non-redundant protein sequence database in Galaxy (Blastx, e-value = 10e-4). We
201 subsequently used the Blast2GO platform to load the resulting Blast-xml file and to conduct
202 mapping and annotation steps based on the BLAST result for acquiring GO annotations for each
203 contig (Götz et al., 2008). To test significantly elevated or decreased dN/dS ratios among genes
204 associated with specific GO terms, we selected those that contained at least 20 orthologs and tested
205 their dN/dS distributions with those of all the observed orthologs (background) using a Wilcoxon
206 test. All statistical analyses were performed in R studio ver. 1.1.453 and *P* values acquired were
207 adjusted by FDR (RStudioTeam, 2016).
208

209 # Results

210 **Performance of four *Pieris* butterflies on 25 Brassicaceae plants.**

211 We obtained larval weights for four *Pieris* butterfly species (*Pieris napi*, *P. melete*, *P. rapae* and *P.*
212 *brassicae*) feeding on 25 different Brassicaceae plant species. Analysis showed that the larval
213 performances of the four *Pieris* species could be clustered into two groups: the *P. napi-P. melete*
214 group and the *P. rapae-P. brassicae* group. The gap statistics for the given number of clusters were
215 as follows: Gap_1 =0.080, Gap_2 = 0.135, Gap_3 = 0.119, Gap_4 = 0.123 (Fig. 3). For instance, we
216 observed that *P. napi* and *P. melete* performed better on *Arabis hirsuta* or *Turritis glabra,* whereas
217 *P. rapae* and *P. brassicae* did better on *Thlaspi arvense* than the other two species (Fig. 3). In
218 addition to this trend, the larvae of four *Pieris* butterfly species also performed similarly. We
219 observed that all four *Pieris* species performed better on *Cardamine occulta* than on the other plant
220 species tested and did not perform well on *Erysimum cheiranthoides* or *Berteroa incana* (Fig. 3).

221

222 **RNA-seq, reciprocal best hit (RBH) BLAST analysis of *Pieris* butterflies**

223 We obtained 32-40 million Illumina 100 bp pair-end reads for the four species (*P. napi*, *P. melete*,
224 *P. rapae* and *P. brassicae*) and 64 million Illumina 150 bp pair-end reads for *P. canidia*. *De novo*
225 transcriptome assemblies using Trinity resulted in 64,279; 62,054; 59,327; 53,004; and 149,481
226 contigs, and in N50 values of 2,048 bp; 2,132 bp; 2,060 bp; 2,594; and 2,075 bp for *P. napi*, *P.*
227 *melete*, *P. rapae P. brassicae*, and *P. canidia* respectively. Using RBH BLAST on the five *Pieris*
228 species, we obtained transcriptome data resulted in 2723 ortholog sets.

229

230 **Identifying signatures of selection on NSP-like gene family members**

We calculated dN/dS ratios for all ortholog sets in the 10 *Pieris* species pairs used with PAML 4.8 (Yang, 2007) and averaged these values. The complete distribution of averaged dN/dS values is shown in Figure 4 (mean dN/dS = 0.10486). The averaged dN/dS values of NSP-like gene family members are as follows: $dN/dS_{NSP} = 0.324$, $dN/dS_{MA} = 0.188$ and $dN/dS_{SDMA} = 0.125$. The dN/dS value of NSP is located in the top 2.72% of the entire dN/dS distribution, whereas MA and SDMA values are lower (MA 11.4%, SDMA 23.4%). We also found a similar pattern between species, where NSPs were in the top 5% in 5 pairs out of 10 (*napi – rapae, napi – canidia, melete – rapae, melete – canidia, rapae – canidia*) and in the top 5.5% in two pairs (*napi – brassicae, melete – brassicae*); MAs and SDMAs were not ranked in the top 5 % (Fig. 5). In most cases, NSPs had the highest value, MAs had higher dN/dS value compared to SDMAs, and the order of dN/dS values of NSP-like gene family members was NSP > MA > SDMA in 8 out of 10 species pairs (Fig. 5).

Signature of clade-specific positive selections on NSP associated with larval performance

We reconstructed Japanese *Pieris* phylogeny using the transcriptome data by concatenating all RBH ortholog sets. The obtained results showed a highly supported *P. napi–P. melete* clade, and *P. rapae–P. canidia* clade (Fig. 5). We performed the branch-site model approach by codeml and found a signal of positive selection on NSPs at the *P. napi–P. melete* branch (FDR adjusted $P = 0.0178$, LRT), however, we found no sign of positive selection at other branches or in MA or SDMA genes (Fig. 5, Table 1). The BEB analysis suggested that two codon sites had signs of positive selection in NSPs in this branch (Table 1, posterior probability > 0.9). These sites were located in second and third domains of NSPs (position 421 and 503 in the amino acid sequence) and close to the positively selected sites identified in previous work (positions 379 and 523) (Heidel-Fischer et al., 2010). The aBSREL analyses also detected a signature of positive selection on NSP genes only

at the *P. napi–P. melete* branch (FDR adjusted $P = 0.010$), whereas no branch-specific positive selection was detected in MA and SDMA genes (Table 2).

GO terms with elevated dN/dS ratios among five *Pieris* butterflies

After GO annotations of all *P. rapae* RBH contigs, we obtained 1457 GO terms in our datasets. These included 680 related to biological process, 540 to molecular function, and 237 to cellular component GO terms. We conducted the Wilcoxon test for the GO terms, which have more than 20 assigned orthologs, and the result revealed that one biological process -- "proteolysis" -- and two processes associated with molecular function -- "hydrolase activity" and "serine-type endopeptidase activity" -- had significantly elevated dN/dS values when compared to the entire dN/dS distribution of all contigs (Fig. 6, Table 3). This test also showed that 13 GO terms had significantly lower dN/dS values in the three categories (Table 3). These lower dN/dS GO terms included "regulation of transcription, DNA-templated," "ribosome biogenesis," and "translation" in biological process; "ATP binding," "structural constituent of ribosome," "GTP binding," "calcium ion binding," "DNA-binding transcription factor activity" and "sequence-specific DNA binding" in molecular functions; and "nucleus," "cytoplasm," "ribosome" and "transcription factor complex" in the category of GO cellular components.

Discussions

Focusing on five Japanese *Pieris* butterflies, we tested host-plant performance and investigated signatures of selection on NSP-like genes, which are a key innovation of these butterflies to overcome the GLS defense system of their Brassicales host plants (Edger et al., 2015; Wheat et al., 2007). We acquired RBH ortholog sets expressed in larvae of the five *Pieris* species based on transcriptome data and compared the calculated dN/dS ratios of each ortholog in order to investigate the effect of evolutionary forces on NSP-like gene family members. We also combined ecological approaches for acquiring performance data on larvae of *Pieris* species by conducting comprehensive feeding experiment using 25 Brassicaceae plant species. These approaches yielded four major findings. First, we observed that *Pieris* species showed clade-specific differences in larval host performance. Second, we observed that NSP genes had significantly elevated dN/dS ratios compared to other genes in the five *Pieris* species, including members of the same gene family, MAs and SDMAs. Third, evidence of positive selection on NSPs was observed at a phylogenetic branch which showed differences in larval performance according to our feeding assays. Last, we observed significantly elevated dN/dS ratios in GO terms which are associated with potential detoxification-related genes in *Pieris* larvae.

According to our feeding experiments with four Japanese *Pieris* species (*P. napi*, *melete*, *rapae* and *brassicae*) and 25 Brassicaceae plant species, *P. napi* and *P. melete* larvae performed similarly, as did *P. rapae* and *P. brassicae* larvae (Fig. 3). Observations in the field suggest that these four *Pieris* species have slightly different host preferences: *P. napi* and *P. melete* feed on wild and montane Brassicaceae plants, such as *Arabis* or *Turritis*, and *P. rapae* and *brassicae* use

295 Brassicaceae crops more often than the other two species (Fig. 1) (Harvey, Poelman, & Gols, 2010;
296 Ohsaki & Sato, 1994). Thus, our results confirm the field observations (Fig. 3). Phylogenetic
297 analysis showed that the *P. napi* and *P. melete* clade was strongly supported, and the species
298 phylogeny seemed to correspond with larval performance (Fig. 5), suggesting that the larval host
299 preferences of the four *Pieris* butterflies are phylogenetically conserved. In this study, we did not
300 perform any physical or chemical defense analyses on the different Brassicaceae plants species we
301 used; however, a number of previous studies revealed that the GLS profiles of Brassicaceae plants
302 can differ dramatically among Brassicaceae species (Agerbirk & Olsen, 2012; Fahey, Zalcmann,
303 & Talalay, 2001; Olsen et al., 2016). Our results suggest that *Pieris* species might not always be
304 capable of fully adapting to the defenses in the ranges of their potential host plants and so likely
305 evolved to feed on a subset of Brassicaceae plants.
306
307 Comparing averaged dN/dS ratios among all species pairs for each ortholog, we found
308 that only NSPs had higher dN/dS values among NSP-like gene family members (Fig. 4). Although
309 we filtered out a number of genes by RBH processes and therefore compared only a subset of the
310 entire orthologs, our finding strongly suggests that NSPs are under positive selection – or, more
311 relaxed purifying selection -- among the five *Pieris* butterfly species. In our interspecies dN/dS
312 comparison, we also observed that NSPs had higher dN/dS values than the other ortholog sets in
313 most of the species pairs (they were located in the top 5.5% in 7 out of 10 species pairs), supporting
314 the hypothesis of positive selection on NSPs in this genus (Fig. 5). In previous research, which
315 calculated dN/dS values from partial NSP sequences of *P. rapae* and *P. brassicae* with 70 other
316 genes, higher dN/dS values of NSPs were observed (dN/dS = 0.25 ranked in the top 5%) (Heidel-
317 Fischer et al., 2010). We found that dN/dS values in our dataset from entire NSP mRNA sequences

318 of this species pair were 0.257, which ranked in the top 6.06 %, thus supporting previous findings.
319 Interestingly, we also found that in most cases MAs had lower dN/dS values compared to NSPs (in
320 both averaged dN/dS ratios and interspecies comparisons) (Figs. 4, 5), and their dN/dS values did
321 not reach the top 5%, suggesting that in this genus, MAs are under stronger purifying selection
322 than are NSPs. NSPs and MAs are known as paralogs, and only NSP was confirmed to have GLS-
323 disarming activity in *Pieris.* However, MAs also disarm GLSs in another Brassicaceae-feeding
324 Pierid genus, *Anthocharis*, which has only MAs (Edger et al., 2015); this overlap strongly suggests
325 that in *Pieris* MAs act like NSPs. Our results show that selection on these two paralogous genes,
326 both of which have similar structure and can potentially disarm GLSs, can differ strikingly. This
327 could imply that these paralogs have differentially functionalized in *Pieris,* where NSPs have more
328 derived functions, whereas MAs have more conservative functions. DN/dS values of SDMAs were
329 lowest among all NSP-like gene family members and also had similar values compared to the
330 average of all the orthologous sets. This similarity suggests that SDMAs are under strong purifying
331 selection and have a conserved function in *Pieris.* Expressed in the gut, SDMAs are known to be
332 found in all Lepidoptera, supporting the hypothesis that their function is related to digestion
333 (Fischer et al., 2008; Randall et al., 2013).
334
335 Using the branch-site model analysis by codeml, we detected evidence of positive
336 selection only in NSPs at the *P. napi-melete* branch (Table 1). Testing all possible branches of all
337 NSP-like gene family members with aBSREL, we detected signatures of positive selection in NSPs
338 only at this branch (Table 2). Based on our comprehensive feeding experiment and phylogenetic
339 analyses, we found that the *P. napi-melete* branch had different host preferences from *P. rapae* and
340 *P. brassicae* (Figs. 2, 4). These results suggest that host-plant preferences in *Pieris* were associated

341 with the evolution of NSPs but not MAs or SDMAs. In this study, we did not test the functional
342 differences of NSPs among the five *Pieris* species. Furthermore, we could not determine whether
343 the differences in larval performances that we observed among the four *Pieris* species were caused
344 by the dissimilarity among the GLS profiles of the host plants. However, our findings imply a
345 strong relationship between the evolution of NSPs and host-utilization patterns among *Pieris*
346 butterflies. Moreover, it is also interesting that only NSPs showed this signature of selection,
347 suggesting that NSPs have been functionalized to detoxify GLSs specific to certain plant species;
348 in contrast, MAs may have evolved to disarm the widespread types of GLSs such as are found
349 universally across Pieridae host plants. In addition, we found positively selected sites in the second
350 and third domains of NSPs, and in earlier population genetic work using *P. rapae* (Heidel-Fischer
351 et al., 2010). Although the molecular mechanisms of the GLS-disarming function of NSPs and
352 MAs are still unclear, our results suggest that the second and third domains of NSPs are important
353 for substrate specificity.
354
355 Besides individual NSP gene family members, elevated dN/dS values were also more
356 broadly observed among the five *Pieris* butterflies in several GO categories, including “proteolysis”
357 (biological process), and “serine-type endopeptidase activity” and “hydrolase activity” (molecular
358 function). In Lepidopteran larvae, most of the digestive enzymes are involved in proteolysis (Simon
359 et al., 2015) and several classes of digestive enzymes are necessary for insect herbivores to acquire
360 essential nutrients in appropriate amounts (Broadway, 1989). In *Pieris*, these proteolytic activities
361 were dominated by serine endopeptidases (Broadway, 1996). Since plants also have varied species-
362 specific protease inhibitors to inhibit protease activity in herbivores, herbivores need to have
363 evolved inhibitor-resistant proteinases as a counter adaptation (Bolter & Jongsma, 1997). Our

364 findings showed signs of positive selection in protease-related genes among five *Pieris* species,
365 suggesting that these genes have accumulated more functional changes as a consequence of
366 interactions with plants in their specific host-plant ranges. A number of genes with hydrolase
367 activity are included in genes related to detoxification in herbivores (Simon et al., 2015). Previous
368 research has uncovered differential gene regulation of this GO term member in several herbivore
369 species responding to different host-plants (Schweizer, Heidel-Fischer, Vogel, & Reymond, 2017).
370 Therefore, a sign of positive selection or relaxed purifying selection on this GO member may also
371 be associated with the host-plant spectra in *Pieris* butterflies.
372
373 To uncover the co-evolutionary diversification of plants and herbivores, it is important
374 to understand the molecular interactions between all involved partners. We found the signature of
375 positive selection on NSPs in a Pieridae genus, *Pieris*, associated with respective host-plant usage.
376 It seems that the evolution of host-plant adaptive genes is correlated with patterns of host-plant
377 usage in this *Pieris* butterfly genus. Moreover, we also observed that MAs, which are paralogs of
378 NSPs, are under more strict purifying selection than NSPs. Our findings combine results from
379 genetic and ecological assays to focus on how the evolution of these two paralogous genes may
380 affect the arms-race between Brassicales and *Pieris* butterflies and their consequent diversification.
381 Functional assays focusing on selected sites will increase our understanding of the evolution and
382 functional differentiation of NSPs and MAs and how *Pieris* adapted evolutionarily to diverse
383 glucosinolates in their host plants.
384

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Data Accessibility

The RNA-seq short read data have been deposited in the EBI short read archive (SRA) with the following sample accession numbers: ERX2829492-ERX2829499. The complete study can also be accessed directly using the following URL: http://www.ebi.ac.uk/ena/data/view/PRJEB29048.

Author Contributions

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

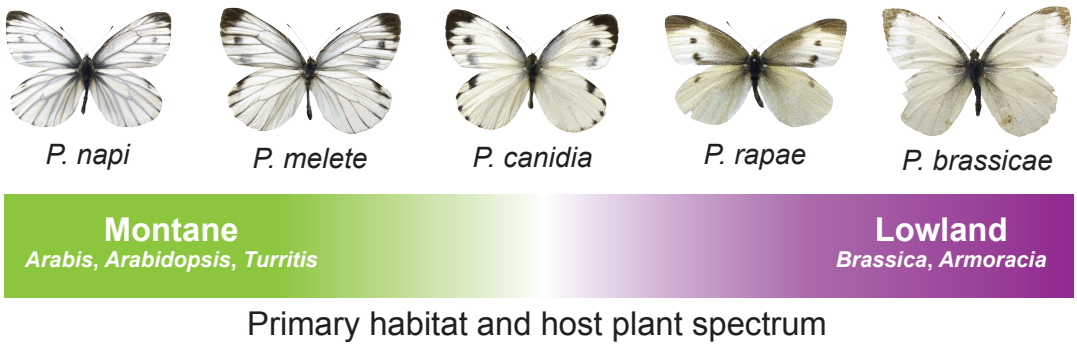

Fig. 1

Field observations of primary habitat and larval host-plant spectra of five *Pieris* butterflies in Japan. *P. napi* and *P. melete* tend to be found in montane habitat and rely mostly on Brassicaceae plants in forests; these include *Arabis*, *Arabidopsis* or *Turritis*. *P. rapae* and *P. brassicae* are known as Brassica crop pests. In Japan, *P. canidia* can only be found in a restricted area and uses *Cardamine* or *Lepidium* as host plants.

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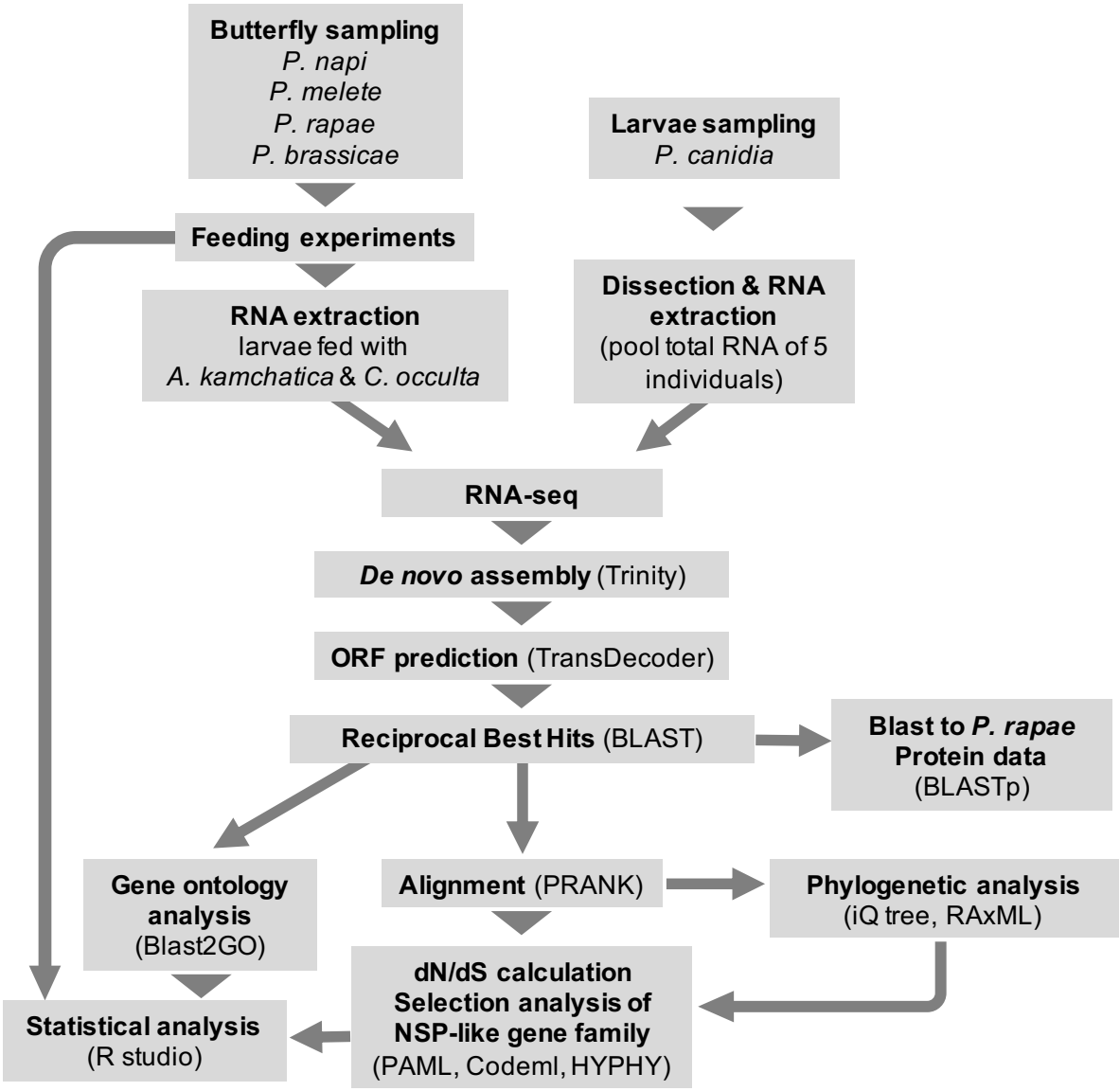

539

540 **Fig. 2**

541 Analysis pipeline used to compare dN/dS ratios of NSP-like gene family members with all
542 observed ortholog sets from the reciprocal best hit using BLAST across five *Pieris* butterflies.
543 Signatures of selection on NSP-like gene family members were investigated in each phylogenetic
544 branch and compared with the results of the feeding assay.

545

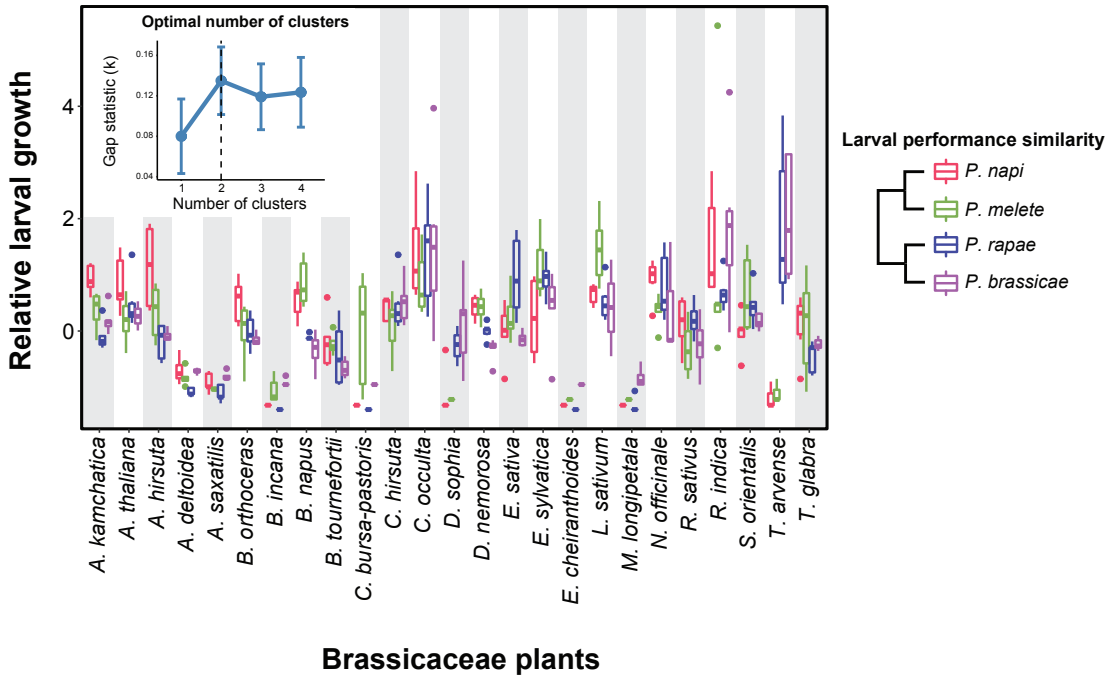

Fig. 3

Feeding assays of four *Pieris* butterfly larvae on 25 different Brassicaceae plants ($n = 6$). The four *Pieris* butterfly species generally grew better on *Cardamine occulta* but could not use *B. incana* or *E. cheiranthoides* as optimal hosts. Overall larval performance patterns of the four *Pieris* species could be clustered in two groups: *P. napi – melete* and *P. rapae – brassicae*. *P. napi* and *P. melete* larvae grew better on *Arabis hirsuta* or *Turritis glabra*, and *P. rapae* and *P. brassicae* larvae grew better on *Thlaspi arvense*.

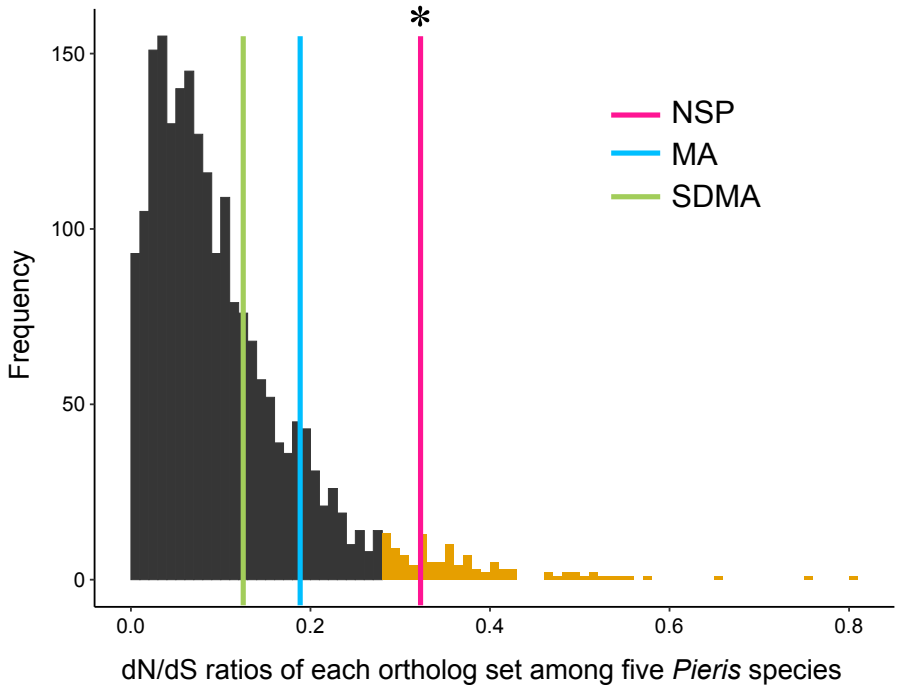

Fig. 4

The averaged distribution of dN/dS values within each ortholog among the five *Pieris* species (n = 2723). The top 5% values in the histogram are colored orange. The vertical lines show dN/dS values of NSP-like gene family members; NSP (pink) = 0.324, MA (blue) = 0.188 and SDMA (green) = 0.125. '*' shows the line is in the top 5%. The dN/dS values of NSP are located in the top 5% of the entire dN/dS distribution (NSPs are located in 2.72%), whereas those of MAs and SDMAs are not (MAs 11.4%, SDMAs 23.4%).

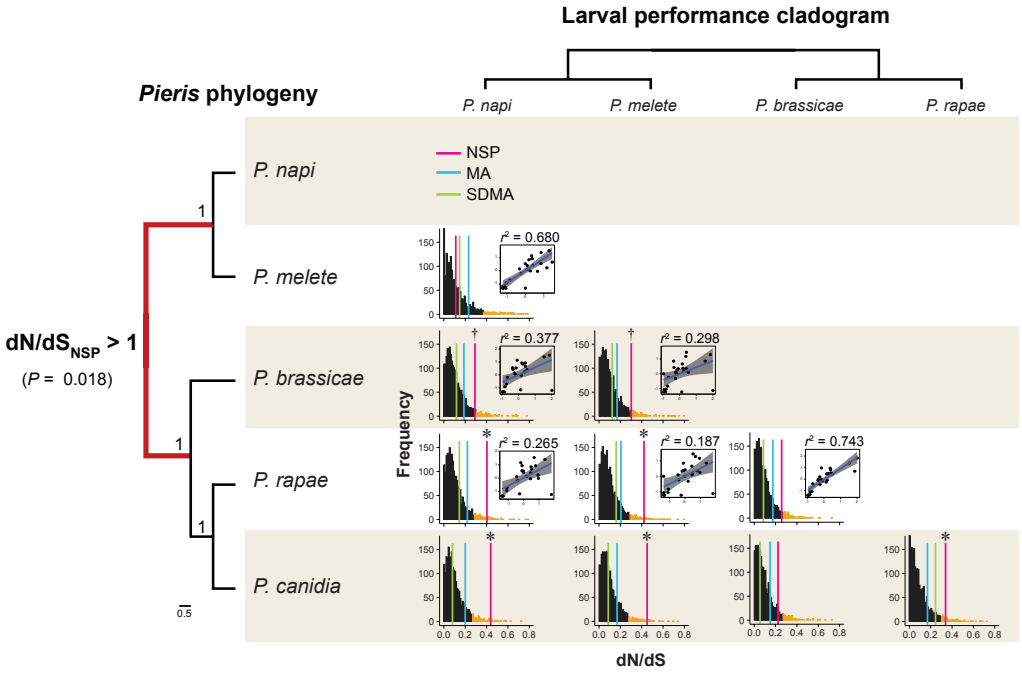

Fig. 5

The species pairwise dN/dS values compared with the results of feeding behavior of *Pieris* butterflies. The histograms showed distributions of species pairwise dN/dS values of entire ortholog sets (showing only from $0 \leq dN/dS \leq 0.8$ for displaying in scale and the top 5% are colored orange). The positions of each NSP-like gene family member are highlighted with colored vertical lines (NSP: pink, MA: blue, SDMA: green). Symbols on the lines show ranking ('*': in the top 5%, '†': in the top 5.5%). The scatterplots show the larval growth of each pair of *Pieris* species except for *P. canidia*. A phylogenetic tree was reconstructed by all the aligned contigs of the five *Pieris* species with bootstrapping support in each node. We found a signature of positive selection in NSPs at the *P. melete* and *P. napi* branch (left phylogeny: $P = 0.018$, LRT), which is also supported by significantly elevated dN/dS values of NSP in the species pairs at the phylogenetic branch (*P. napi* -. *P. rapae, P. napi* - *P. brassicae, P. melete* - *P. rapae, P. melete* - *P. brassicae*). Furthermore, dissimilarities in larval performance correlated with elevated dN/dS values of NSPs.

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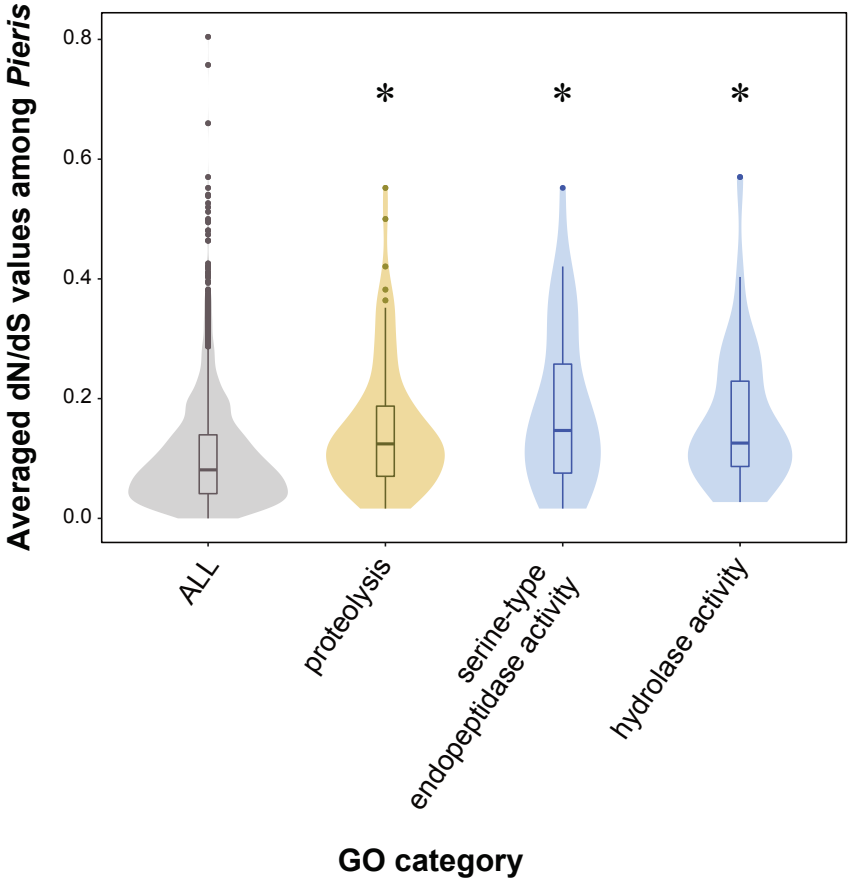

581
582 **Fig. 6**
583 GO terms which have significantly elevated dN/dS values compared to those of entire ortholog sets
584 among *Pieris*. Elevated dN/dS values were observed in "proteolysis" from biological process
585 (orange), and "serine-type endopeptidase activity" and "hydrolase activity" from molecular
586 function (blue) compared to the entire distribution of all the observed contigs among *Pieris* (gray).
587 Comparisons with other enriched GO terms are shown in Table 3. '*': *P* values are adjusted by
588 FDR; $P \leq 0.05$, Wilcoxon test.
589

Tables

Table 1 Branch-specific selection tests on NSP-like gene family by codeml

Gene	Branch name	lnL *Model null*	lnL *Model alt*	delta L	*P* value (FDR adjust.)	BEB site p > 0.9
NSP	***P. napi - melete***	**-3816.12**	**-3811.56**	**9.11**	**0.018**	**421 E 0.918, 503 R 0.960**
	P. rapae - canidia branch	-3816.98	-3816.68	0.6	1	
	P. napi	-3817.87	-3817.87	0	1	
	P. melete	-3817.87	-3817.87	0	1	
	P. brassicae	-3817.87	-3817.87	0	1	
	P. rapae	-3816.44	-3816.44	0	1	
	P. canidia	-3817.87	-3817.87	0	1	
MA	*P. napi - melete* branch	-4173.71	-4172.82	1.77	0.549	
	P. rapae - canidia branch	-4172.77	-4170.79	3.96	0.279	
	P. napi	-4171.43	-4171.43	0	1	
	P. melete	-4173.71	-4173.71	0	1	
	P. brassicae	-4173.71	-4173.71	0	1	
	P. rapae	-4171.88	-4171.88	0	1	
	P. canidia	-4173.71	-4173.71	0	1	
SDMA	*P. napi - melete* branch	-1198.58	-1198.58	0	1	
	P. rapae - canidia branch	-1198.58	-1198.58	0	1	
	P. napi	-1198.57	-1198.57	0	1	
	P. melete	-1198.03	-1196.39	3.28	0.490	123 M 0.910
	P. brassicae	-1198.58	-1198.58	0	1	
	P. rapae	-1196.17	-1196.16	0.02	1	
	P. canidia	-1198.58	-1198.58	0	1	

lnL *Model null*: log likelihood for null model with fixed dN/dS ratios. lnL *Model alt*: log likelihood for alternative model which allows having unfixed dN/dS values at the branch. Delta L: 2(lnL *Model alt* - lnL *Model null*) for the likelihood ratio test (LRT). *P* values are from LRT and adjusted for multiple testing. BEB analysis shows the specific sites which have significant signatures of positive selection with posterior probability. Positions are based on *P. rapae* protein sequences.

Table 2 Branch-specific selection tests on NSP-like gene families by aBSREL

Gene	Branch name	LRT	*P* value
NSP	***P. napi* - *melete* branch**	**10.96**	**0.010**
	P. rapae - *canidia* branch	5.06	0.067
	P. napi	0	1
	P. melete	5.46	0.067
	P. brassicae	2.33	0.208
	P. rapae	0	1
	P. canidia	0	1
MA	*P. napi* - *melete* branch	3.02	0.967
	P. rapae - *canidia* branch	4.43	0.967
	P. napi	0	1
	P. melete	0	1
	P. brassicae	0.96	1
	P. rapae	0	1
	P. canidia	4.27	0.967
SDMA	*P. napi* - *melete* branch	0	1
	P. rapae - *canidia* branch	0.49	1
	P. napi	0	1
	P. melete	4.38	1
	P. brassicae	0	1
	P. rapae	0	1
	P. canidia	0	1

LRT: Likelihood ratio test statistic for selection. *P* values are adjusted by false discovery rates.

Table 3 GO terms with elevated or decreased dN/dS values corresponding to entire orthologs.

ALL: all the orthologs with assigned GO term. N: number of orthologs in the GO term. *P* values are adjusted with false discovery rates. GO terms with elevated dN/dS values are in bold.

GO term	N	dN/dS mean	*P* value*		up/down to ALL
ALL	2113	0.105	-		
Biological process					
oxidation-reduction process	109	0.103	0.514		
proteolysis	**88**	**0.149**	**<0.001**	***	**up**
regulation of transcription, DNA-templated	88	0.070	0.000	***	down
transmembrane transport	60	0.093	0.790		
ribosome biogenesis	39	0.057	0.001	***	down
carbohydrate metabolic process	38	0.105	0.551		
translation	34	0.058	0.002	**	down
signal transduction	28	0.083	0.361		
protein phosphorylation	27	0.071	0.185		
phosphorylation	26	0.115	0.517		
methylation	21	0.130	0.253		
purine nucleobase metabolic process	21	0.087	0.551		
Molecular function					
ATP binding	158	0.075	0.001	***	down
nucleic acid binding	100	0.120	0.237		
zinc ion binding	99	0.110	0.443		
metal ion binding	83	0.123	0.080		
DNA binding	81	0.096	0.295		
RNA binding	72	0.091	0.312		
structural constituent of ribosome	36	0.058	0.002	**	down
oxidoreductase activity	32	0.118	0.112		
hydrolase activity	**32**	**0.164**	**0.002**	**	**up**
GTP binding	32	0.073	0.017	*	down

serine-type endopeptidase activity	**30**	**0.182**	**0.002**	**	**up**
transmembrane transporter activity	29	0.092	0.662		
calcium ion binding	27	0.053	0.002	**	down
DNA-binding transcription factor activity	26	0.064	0.028	*	down
sequence-specific DNA binding	25	0.040	<0.001	***	down
transferase activity	24	0.088	0.408		
ligase activity	24	0.104	0.112		
GTPase activity	22	0.076	0.091		
kinase activity	21	0.117	0.567		
helicase activity	21	0.098	0.848		
methyltransferase activity	20	0.125	0.327		
iron ion binding	20	0.112	0.379		
Cellular component					
integral component of membrane	479	0.103	0.477		
nucleus	164	0.088	0.002	**	down
cytoplasm	64	0.087	0.036	*	down
ribosome	62	0.071	0.017	*	down
extracellular region	41	0.110	0.477		
intracellular	32	0.088	0.477		
transcription factor complex	30	0.063	0.017	*	down
membrane	22	0.124	0.541		
mitochondrion	20	0.109	0.509		

FDR adjusted *P* value: "*" < 0.05, "**" < 0.01, "***" < 0.001