Evolution of Gustatory Receptor Gene Family Provides Insights into Adaptation to Diverse Host Plants in Nymphalid Butterflies

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Abstract

The host plant range of herbivorous insects is a major aspect of insect–plant interaction, but the genetic basis of host range expansion in insects is poorly understood. In butterflies, gustatory receptor genes (GRs) play important roles in host plant selection by ovipositing females. Since several studies have shown associations between the repertoire sizes of chemosensory gene families and the diversity of resource use, we hypothesized that the increase in the number of genes in the GR family is associated with host range expansion in butterflies. Here, we analyzed the evolutionary dynamics of GRs among related species, including the host generalist Vanessa cardui and three specialists. Although the increase of the GR repertoire itself was not observed, we found that the gene birth rate of GRs was the highest in the lineage leading to Vanessa cardui compared with other specialist lineages. We also identified two taxon-specific subfamilies of GRs, characterized by frequent lineage-specific duplications and higher non-synonymous substitution rates. Together, our results suggest that frequent gene duplications in GRs, which might be involved in the detection of plant secondary metabolites, were associated with host range expansion in the Vanessa cardui lineage. These evolutionary patterns imply that the capability to perceive various compounds during host selection was favored during adaptation to diverse host plants.

Key words: gustatory receptor, gene duplication, chemoreception, host range expansion, host plant selection, butterfly.

Introduction

Herbivorous insects comprise more than one-third of described species, and many authors claim that host plant associations have been major forces driving insect species diversification (Ehrlich and Raven 1964; Mitter et al. 1988; Wiens et al. 2015). In particular, the host plant range, which refers to the variety of plant species consumed or used as oviposition sites, is considered an important factor in adaptation of herbivorous insects. Several studies have suggested that host range evolution has facilitated species diversification (Janz et al. 2006; Janz and Nylin 2008; Nylin et al. 2014). According to the “oscillation hypothesis” detailed in Janz and Nylin (2008), expansion of the host plant range in an ancestral species would result in a high speciation rate in the following lineage. However, the evolutionary processes of host range expansion in herbivorous insects are poorly understood, because the genetic basis of host plant adaptation has been largely unexplored in most insect taxa.

Host plant selection by ovipositing females is one of the key determinants of host range (Janz and Nylin 1997). Because the larvae of herbivorous insects generally have low dispersal abilities, adult females have to recognize and lay eggs on plants that are suitable for larval diet. In butterflies, adult females can discriminate their host and non-host plants by touching the surface of the plant with their foreleg tarsi (Renwick and Chew 1994). This drumming behavior allows
butterflies to perceive soluble secondary metabolites on the plant, and adult females decide to oviposit or not based on the chemical blend (Nishida et al. 1987; Pereyra and Bowers 1988; Huang et al. 1993). From these observations, chemosensory gene families have been seen as the biggest candidate genes regulating host selection behavior of butterflies. Although the precise genetic mechanisms underlying the preference for specific hosts for oviposition are yet to be elucidated, Ozaki et al. (2011) showed that a gustatory receptor gene (GR) encoded a receptor for a host plant-specific compound (i.e., synephrine), and regulated the oviposition behavior of the Asian swallowtail butterfly, Papilio xuthus. Moreover, Briscoe et al. (2013) revealed that many GR genes had female-biased expression patterns in the postman butterfly, Heliconius melpomene, suggesting the importance of GRs for host selection by females. Because drumming behavior is widely observed among butterflies, GRs likely play essential host selection roles in other butterfly taxa, which also influences host range expansion.

GRs comprise one of the biggest gene families in insect genomes. This gene family evolved under the birth-and-death model, in which gene repertoires are shaped by multiple gene gains (duplications) and losses (deletions or pseudogenization), and the gene number varies among lineages (Nei et al. 2008; Sánchez-Gracia et al. 2009). Several studies have reported possible associations between the repertoire sizes of the GR family and the varieties of resource use in insects. In Drosophila, for example, two specialist species have fewer numbers of GRs than their non-specialist sister species because of accelerated gene losses, which were apparently caused by relaxed selection after specializations on chemically homogeneous environments (McBride 2007; McBride et al. 2007). Differences in the number of GRs have also been observed in the other direction, that is, host generalists: recent studies on genome sequences of extreme polyphagous herbivores in the family Noctuidae, such as the cotton bollworm moth Helicoverpa armigera and the tobacco cutworm Spodoptera litura, revealed that they have remarkably larger GR repertoires in the genomes compared with other lepidopteran host specialists (Xu et al. 2016; Cheng et al. 2017; Gouin et al. 2017; Pearce et al. 2017). These observations raise the possibility that evolutionary transition from host specialist to generalist in butterflies is accompanied by the increase in the number of GRs by gene duplications. However, the evolutionary relationships between the GRs and host expansion remain uncertain, because previous works did not present genomes for specialist species in Noctuidae, andcline distances of 25°C, 16L/8D until eclosion. Three female individuals of each species had all their legs dissected within 2 days of eclosion. The legs of each individual were separately homogenized as soon as possible after dissection, and the total RNA was extracted from the homogenates using a Maxwell 16 LEV Plant RNA kit (Promega Corporation, WI, USA) following the manufacturer’s protocol. The qualities of total RNA samples were measured using an Agilent 1000 Bioanalyzer (Agilent Technologies, CA, USA).

RNA-Sequencing and De Novo Assembly

Although detection and comparison of the gene repertoires should be based on whole genome sequences, they were not
available for our study species. Instead, we applied RNA-seq and de novo assembly to identify candidate genes. As insect GRs generally have very low expression levels (Clyne et al. 2000; Ozaki et al. 2011), we focused on sequencing efforts on female legs, in which the most diverse GRs are expressed among body parts of the butterfly (Briscoe et al. 2013), rather than sequencing all tissues evenly at low-sequencing depths. Our strategy for RNA-seq was to combine 100 bp and 300 bp paired-end reads obtained using HiSeq 2500 and MiSeq sequencers (Illumina, CA, USA), respectively, to obtain the longest possible gene sequences. Briefly, paired-end cDNA libraries were constructed using 500 ng of total RNA from each butterfly obtained using the TruSeq RNA Sample Prep Kit v2 (Illumina) according to the manufacturer’s protocol. Three libraries were obtained for the HiSeq system and one library for the MiSeq system from each species (see supplementary fig. 1, Supplementary Material online) for a schematic explanation). All RNA-seq runs were conducted at NODAI genome research center (Tokyo, Japan) between 2015 and 2016, except for a MiSeq library of A. burejana that was sequenced at JT Biohistory Research Hall (Osaka, Japan) in 2016.

Low-quality raw RNA-seq reads were removed using the fastq_quality_filter of FASTX-toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit; last accessed May 19, 2018), with a setting of -V -Q 33 -q 20 -p 30. Processed reads were assembled using Trinity 2.1.1 (Grabherr et al. 2011) with the --trimmomatic option on the default settings. All the reads from both HiSeq and MiSeq data were collectively assembled (supplementary fig. 1, Supplementary Material online). Qualities of de novo assemblies were listed in supplementary table 1, Supplementary Material online.

Gene Homology Search, Phylogenetic Analysis, and Gene Model Construction

To detect candidate GR genes from the Trinity assemblies, TBLASTN searches (e-value = 1e-05) (https://blast.ncbi.nlm.nih.gov/Blast.cgi; last accessed May 19, 2018) were performed using the amino acid sequences of GRs of B. mori (Guo et al. 2017), Danaus plexippus (Zhan et al. 2011), and H. melpomene (Briscoe et al. 2013) as input queries and our Trinity assemblies as databases. Contig profiles selected in TBLASTN were further tested with BLASTX (e-value = 1e-02) against the NCBI non-redundant protein database (nr). Contigs that matched GRs of other insect species in BLASTX were considered to be candidate GR genes of the focal species. For contigs having multiple isoforms, we chose the longest contig as the representative sequence. Amino acid sequences of candidate GR contigs were predicted using TransDecoder (https://transdecoder.github.io; last accessed May 19, 2018) and Sequence Manipulation Suite (Stothard 2000). Subsequently, we repeated the same procedure using amino acid sequences of the candidate GR contigs as the query for TBLASTN to search for contigs that were not detected in the first round.

The amino acid sequences of the GR genes of our study species (V. cardui, V. indica, P. c-aureum, and A. burejana) and those of five other insect species (H. melpomene, Briscoe et al. 2013; D. plexippus, Zhan et al. 2011; P. xuthus, Ozaki et al. 2011; B. mori, Guo et al. 2017; and Drosophila melanogaster, Robertson et al. 2003) were aligned using MAFFT 7.273 (Katoh and Standley 2013) with the L-INS-i algorithm. An initial gene phylogeny was constructed using the maximum likelihood method in RAxML 8.2.8 (Stamatakis 2014), followed by bootstrap analyses with 100 replications. We utilized “-rm PROTGAMMAAUTO” option to identify the most suitable protein substitution model, and JTT model was assigned.

At this point, however, we observed many cases where one GR gene of the nymphalid species seemed to be fragmented into several contigs in the gene phylogeny. To avoid overestimation of the number of GRs, we searched for contigs that could be integrated into a single gene model. We first identified sets of orthologous genes occupying close positions in the phylogeny. Nucleotide sequences of each gene set were then aligned using MAFFT. Here, we set specific criteria to determine whether the multiple contigs originated from a single gene: 1) If multiple contigs of one species aligned to their orthologous genes without overlaps between contigs, they were combined into a single gene model. The contig identities were later confirmed by PCR and electrophoresis (Supplementary Material online). For a few gene models, sequence gaps were determined by Sanger sequencing. 2) If multiple contigs of the species overlapped in alignments, we calculated synonymous substitution rates (dS) in the overlapping regions, following the method used in Duncan et al. (2014). If dS was <0.19, we collapsed the contigs into a single gene model (i.e., contigs were considered as alleles). Otherwise, we counted those contigs as paralogs. As dS is often considered as an index for divergence times between homologs (Lynch and Conery 2000), what we tried here was collapsing contigs with shorter divergence times. The cut-off value for dS (0.19) was average pairwise dS between V. cardui and V. indica, calculated from 30 randomly chosen BUSCO genes using codeml in PAML v4.8 (Yang 2007) (supplementary table 2, Supplementary Material online). Because of this methodology, we had to repeat the phylogeny construction process and gene alignment tests until no contigs remained that could be combined with others. Hereafter, we considered each gene model as a unit of GR gene.

After obtaining the final set of GRs, a final gene phylogeny was constructed using the maximum likelihood method in RAxML, with JTT substitution model assigned by “PROTGAMMAAUTO” option. Bootstrap analyses were further carried out with 500 replications. The phylogeny was visualized using iTOL v3 (Letunic and Bork 2016). Based on ligand information of several insect GRs from previous studies, we inferred the ligand affinities of each phylogenetic subfamily.
As chemoreception plays a major role in host selection of butterflies, we also explored other chemosensory gene families expressed in female legs. Homology searches and constructions of gene models were also performed for olfactory receptor (OR), ionotropic receptor (IR), odorant-binding protein (OBP), and chemosensory protein (CSP) gene families in the same manner as GRs.

Comparisons of the Number of GRs
The difference in the number of GRs (i.e., the number of GR gene models) among species was determined. First, we counted the numbers of insect-specific BUSCO (single-copy orthologs conserved among the lineage) genes (v3, Insecta) (Simão et al. 2015) in each assembly to consider the variation in the number of genes due to the de novo assembly procedure (supplementary table 3, Supplementary Material online). We calculated the ratio of the number of GRs to that of BUSCO in each assembly and tested the heterogeneity of the frequency among species using the pairwise Fisher test with the Benjamini–Hochberg correction (Benjamini and Hochberg 1995). Other chemosensory genes detected were also analyzed in the same procedure.

Estimation of GR Gain/Loss Events and Birth/Death Rates
We estimated the numbers of gains and losses of GRs along the species tree using Notung 2.8.1.7 (Stolzer et al. 2012), which reconciles a gene phylogeny onto a species tree. Input species tree was manually drawn based on phylogenetic information of species reported in previous studies (Wahlberg 2006; Wahlberg et al. 2009, 2013; Wahlberg and Robinoff 2011). We used the “—phylogenomics” command implemented in Notung to estimate counts of gains (duplications) and losses (deletions or pseudogenization) of GRs occurring in each phylogenetic branch. We then tested the difference in the ratio of the number of gains and losses among the most recent branches for each species using the pairwise Fisher test with the Benjamini–Hochberg correction (Benjamini and Hochberg 1995). The same analysis was also performed for OR and IR gene family.

We then estimated the gene birth rate (ß) and the gene death rate (δ), defined as the number of gene gains or losses per million years per gene, for each phylogenetic branch after the common ancestor of Nymphalini. Divergence times of the common ancestor of Nymphalini. Divergence times of branch, we then estimated the number of GRs (i.e., the number of GR expression events) using the formula developed by Niimura et al. (2014) (supplementary table 5, Supplementary Material online).

However, we admit our analysis of gene gains and losses based on leg transcriptomic data may be inaccurate, because genes exclusively expressed in other tissues or at other developmental stages would have been ignored from the analysis. In this case, the number of gene gains would be underestimated, and the number of losses would be overestimated. Nevertheless, we believe that the overall pattern of gains and losses was reliable for several reasons. First, legs are one of the primary gustatory-sensing organs for butterflies, thus we assumed that the numbers of GRs expressed in female legs could be used as substitutes for the numbers of the whole GR repertoires. In fact, it has been shown that female legs of H. melpomene expressed the most diverse set of GRs among its body parts (50 out of 73 total GRs, 68%) (Briscoe et al. 2013). The positive relationships between the number of expressed genes at a tissue and the total repertoire size have been reported in ORs. For example, D. melanogaster expressed 44 out of 62 total ORs (70%), and female H. melpomene expressed 67 out of 70 total ORs (96%) only in the antennae, the primary olfactory organ of insects (Couto et al. 2005; Briscoe et al. 2013). Although phylogenetically distant from insects, similar patterns were found for vertebrate ORs expressed at olfactory epithelium, the most important olfactory organ for mammals: 295 out of 366 ORs (80%) and 817 out of 1,154 ORs (70%) were detected from human and mouse, respectively (Zhang et al. 2004, 2007). In contrast, the relationship may not be straightforward for GRs. In S. litura, the most GR-rich tissue was larval maxilla, but it expressed only 84 out of 237 total GRs (35%) (Cheng et al. 2017). Adult legs possessed only 20 GRs (8%) (Cheng et al. 2017). However, these percentages might have been underestimated, because only 123 GRs were detected in their transcriptome analysis across all tissues of larvae and adults (Cheng et al. 2017). Taken together, these observations imply that the number of expressed chemosensory genes at an organ is generally influenced by the size of total gene repertoires, but the extent to which it reflects the total repertoires would depend on the importance of chemoreception at the organ. At least, we expect the numbers of expressed GRs in butterfly legs reflect high proportions of the whole repertoire sizes, as leg chemoreception is commonly important among butterflies. Second, the gene gain/loss estimation by Notung is based on the topology of the input gene phylogeny (Stolzer et al. 2012). Since we collected complete GR repertoires from several species, our final GR gene phylogeny, in general, was highly supported by bootstrap analysis. Therefore, we assume that the topology of the phylogeny would not dramatically change even if missing GRs of Nymphalini were included, and the overall pattern of gene gains and losses would not be affected. Further analysis using complete GR gene repertoires needs to be performed to verify this assumption.

Evolutionary Rate Analysis
We took two approaches to infer dS/dD ratios across the GR family. First, we chose pairs of orthologous GRs between...
V. cardui and V. indica, and between V. cardui and A. burejana, based on phylogenetic positions on the GR gene phylogeny. If a GR gene had a one-to-many relationship with its orthologs, we considered each different combination as one pair. In total, we obtained 24 orthologous pairs between V. cardui and V. indica (supplementary table 6, Supplementary Material online), and 26 pairs between V. cardui and A. burejana (supplementary table 7, Supplementary Material online). After nucleotide alignments using MAFFT, we calculated pairwise dN/dS ratios with codeml in PAML 4.8 (Yang 2007). Second, we searched for orthologous gene groups consisting three species (V. cardui, V. indica, and A. burejana as the outgroup), and aligned those nucleotide sequences within groups. When one-to-many genes were included in the group, each different combination was considered as an original sample. We obtained 14 orthologous groups (OGs) in total (supplementary table 8, Supplementary Material online). dN/dS ratios for each branch in the unrooted three-species tree were calculated using codeml under branch model (Yang 2007). The estimation based on branch model is likely to be more accurate than pairwise, but a problem in our data set was that phylogenetic relationships of Nymphalini GRs were so complicated it was difficult to find clear ortholog groups among three species. Therefore, we integrated pairwise dN/dS estimation to cover across the GR phylogeny. Most GRs in our data set were partial sequences, and consequently, the analysis was performed only in aligned regions. We then tested the variation of mean dN/dS ratios using one-way ANOVA for phylogenetic subfamilies and using t-test between one-to-one and one-to-many pairs, respectively. For three-species OGs, we also tested the difference in selective pressures between the V. cardui lineage and the V. indica lineage using paired Wilcoxon test. Any genes showing dS < 0.01 were excluded from the analysis.

Additionally, we searched for signatures of gene conversion among homologous genes of Nymphalini using GENECONV (Sawyer 1989), as gene conversion can modify the estimation of evolutionary rates. Protein sequences alignments of more than three genes (paralogs or homologs in the close positions) were created by MAFFT 2.723 (Katoh and Standley 2013) and used as input data. For genes in which gene conversion were detected, we re-estimated pairwise dN/dS ratios after removing the region of putative conversion from the alignments.

### Results

**Detection and Comparisons of the Numbers of GRs**

Although a variation of total assembled bases was observed, our de novo assemblies exhibited generally similar qualities among four species in terms of N50, average contig length, and the numbers of BUSCO (supplementary tables 1 and 3, Supplementary Material online). The number of GRs found in V. cardui, V. indica, P. c-aureum, and A. burejana were 50, 27, 17, and 45, respectively (tables 1 and 2). For the other chemosensory gene families, we did not observe any significant differences in the number of genes between V. cardui and other specialists (tables 1 and 2).

**GR Gains and Losses along the Nymphalini Phylogeny**

It was estimated that losses of GRs occurred more frequently than gains in most branches in the Nymphalini lineage (fig. 2 and table 3). In contrast, the most recent branch leading to
Table 3
The Estimated Numbers of Gains and Losses in the GR Family, Along With Gene Birth Rates (β) and Death Rates (δ), Among the Most Recent Branches of Four Species.

| Species          | Vanessa cardui | Vanessa indica | Polygonia c-aureum | Araschnia burejana |
|------------------|----------------|----------------|---------------------|---------------------|
| Host Range       | Generalist     | Specialist     | Specialist          | Specialist          |
| No. of Gains     | 7              | 1              | 0                   | 3                   |
| No. of Losses    | 9              | 25             | 40                  | 24                  |
| Gain/Loss ratio  | 0.778          | 0.04           | 0                   | 0.125               |
| Birth rate (β)   | 0.00536        | 0.00101        | 0                   | 0                   |
| Death rate (δ)   | 0.00689        | 0.02519        | 0.02853             | 0.00793             |

V. cardui exhibited a relatively high GR gain/loss ratio (seven gains/nine losses) (fig. 2 and table 3). Comparison of GR gain/loss ratios among the most recent branches for each species revealed that the gain/loss ratio in V. cardui was significantly higher than those of the other species (pairwise Fisher test; P < 0.01 for V. cardui–V. indica; P < 0.001 for V. cardui–P. c-aureum; P < 0.05 for V. cardui–A. burejana) (table 4). We then estimated the gene birth and death rate at each phylogenetic branch. The branch leading to V. cardui showed the highest birth rate (β = 0.00536), whereas the death rate was similar to that of the branch leading to A. burejana (V. cardui: δ = 0.00689, A. burejana: δ = 0.00793) (table 3 and supplementary table 5, Supplementary Material online). However, expansion of the repertoire itself during host range expansion was not observed (from 52 to 50 GRs, fig. 1). The same analysis for OR and IR families did not indicate any lineage-specific accelerations of gene gains or losses (supplementary fig. 3, Supplementary Material online).

Evolutionary Rate Analysis
We estimated pairwise dN/dS ratios between orthologous genes, and those in lineages of V. cardui and V. indica based on the three-species tree, to examine patterns of selective pressures acting on GRs among phylogenetic subfamilies (i.e., sugar, fructose, CO2, LS, and BS). For the pairwise analysis, significant differences in dN/dS were observed among subfamilies in both combinations of species (one-way ANOVA; P < 0.05 for V. cardui–V. indica; P < 0.01 for V. cardui–A. burejana) (fig. 3). Particularly, LS and BS subfamilies showed higher dN/dS ratios, whereas sugar, fructose and CO2 receptor genes exhibited very low evolutionary rates (fig. 3). Moreover, GRs in one-to-many relationships had higher dN/dS ratios compared with one-to-one genes in both combinations (t-test; P < 0.01 for V. cardui and V. indica; P < 0.05 for V. cardui and A. burejana) (fig. 3). The variation of dN/dS estimated for lineages of V. cardui and V. indica showed similar patterns in terms of mean values (fig. 3). However, the differences in dN/dS among subfamilies and between one-to-one and one-to-many genes were not significant for the V. cardui lineage, mainly because of low sample coverage and a few outlier genes with dN/dS > 2 (fig. 3). Although the mean dN/dS was higher in the V. cardui lineage than in V. indica, there was no significant difference in evolutionary rates.

Phylogenetic Analysis
Ligand information of several GRs could be estimated from the gene phylogeny because they were included in the same subfamilies as GRs whose ligands had been reported in past studies, namely, CO2 (Kwon et al. 2007), sugar (Dahanukar et al. 2007), and fructose receptors (Sato et al. 2011) (fig. 2). These subfamilies were mainly characterized by one-to-one orthologous relationships among distantly related species (fig. 2).

Two major taxon-specific GR subfamilies were observed in the gene phylogeny (fig. 2). One comprised nearly half of all the genes, exhibiting frequent lineage-specific expansions. This subfamily only included GRs of Lepidopteran species (i.e., no Drosophila GRs), and was thus named the “Lepidoptera-specific” (LS) subfamily. The other smaller subfamily only contained GRs of butterfly species and was designated the “butterfly-specific” (BS) subfamily (fig. 2). This subfamily originated from the fructose receptor clade, but, unlike the sugar and fructose subfamilies, it was characterized by several lineage-specific gene expansions. Putative species-specific gene duplications in the most recent branches of Nymphalini, including seven putatively duplicated genes in the V. cardui lineage, were mapped on either LS or BS subfamilies (fig. 2).
between orthologs of these species (supplementary table 8, Supplementary Material online).

We detected a possible gene conversion between VindGR23a and VindGR23b (supplementary table 9, Supplementary Material online). To examine the effect of gene conversion, we calculated pairwise \(d_{NS}/d_{SN}\) ratios for these genes after removing regions of the putative gene conversion. As a result, estimated \(d_{NS}/d_{SN}\) ratios were slightly higher without conversion than original values (supplementary table 9, Supplementary Material online).

Discussion

The genetic basis underlying the transition from specialist to generalist has been unexplored in herbivorous insects. In butterflies, GRs play important roles in host plant selection by ovipositing females (Ozaki et al. 2011). It has been suggested that the repertoire sizes of this gene family are associated with the diversity of resource use (McBride et al. 2007; Xu et al. 2016; Cheng et al. 2017; Pearce et al. 2017). Thus, we hypothesized that evolutionary events in the GR family, particularly the increase of the repertoire by gene duplications, were associated with adaptation to diverse host plants by butterflies. To test this hypothesis, we investigated characteristics of GRs among four closely related species from the tribe Nymphalini, including the generalist V. cardui and three specialists. Frequent Gene Duplications of GRs Were Associated With Host Range Expansion

We observed that the gain/loss ratio was particularly higher at the branch leading to V. cardui than the other branches within Nymphalini (fig. 1). Moreover, the gene birth rate was highest at the V. cardui branch, whereas the death rate was not clearly different from those in the other branches (table 3). These findings imply that gene duplications in the GR family occurred frequently in the course of host range expansion, which is consistent with our hypothesis. However, as Notung simply considers clusters of conspecific genes in the phylogeny (i.e., genes in one-to-many relationships) to be species-specific gene duplication events, it was uncertain whether those one-to-many genes represented actual duplications. Nevertheless, the results showed that one-to-many orthologous pairs had higher \(d_{NS}/d_{SN}\) ratios than one-to-one pairs (fig. 3), which is consistent with the observation that duplicated chemoreceptor genes have higher evolutionary rates than non-duplicated homologs (Gardiner et al. 2008; Almeida et al. 2014). Therefore, we expect one-to-many genes in our data set represented recent duplication events in the GR family.

In contrast, the expansion of the GR repertoire size itself was not observed along with the increased rate of gene duplication (fig. 1). Although this result was not consistent with our hypothesis, we note that it was largely influenced by our study design using partial GR repertoires, in which many GRs were likely to be missing. The algorithm of Notung predicts the number of genes at each node as the number of OGs retained in at least one species below the node (Stoizer et al. 2012). For example, the estimated gene number at the common ancestor of V. cardui and V. indica is the sum of the numbers of OGs found only in V. cardui, those found only in V. indica, and those found in both species. Therefore, if partial gene repertoires are placed at the tips, there would be a strong bias that the gene numbers at nodes tend to be larger than those at tips, regardless of the actual changes in
Rather, we would like to focus on the fact that the increase in gene birth rate was observed despite the bias toward gene loss. Changes in the GR repertoire sizes before and after host range expansion should be further addressed in whole genome-based studies.

Another concern is that the variation of the GR repertoire sizes between specialist and generalist was not entirely consistent with previous studies (Xu et al. 2016; Cheng et al. 2017; Gouin et al. 2017; Pearce et al. 2017). Specifically, the number of GRs detected from the generalist V. cardui was significantly larger than V. indica and P. c-aureum, but not different from A. burejana. Again, this could be caused by the use of partial GR repertoires: Even if there is a significant difference in the repertoire sizes at the whole genome scale, that difference could be reduced when we extracted only genes expressed in female legs. Another explanation is that A. burejana lineage, the most distant branch from V. cardui, was influenced by other factors that maintained its GR repertoire relatively large. Besides host range, genome size was proposed as a variable explaining the variation of chemoreceptor gene repertoire sizes among Drosophila (Gardiner et al. 2008), which could also be affecting nymphalid butterflies. To our knowledge, however, there is no evidence showing that genome size is significantly different among our study species, thus we could not reach any conclusion about the relatively large GR repertoire for A. burejana. At least, the fact that lineages for V. cardui and A. burejana can be clearly separated by the gene birth rates supported the importance of GRs during host range expansion.

Because we used only a single generalist species, it was difficult to identify whether the higher rate of gene duplications in GRs was specifically associated with host plant range. However, several studies suggest that there could be associations between gene duplications and adaptation to diverse environments in various organisms. For example, species that survive under a variety of climatic conditions tend to have higher proportions of duplicated genes in the genomes among Drosophila (Makino and Kawata 2012) and mammals.
In the soil bacteria genus *Frankia*, metabolism-related genes were expanded by gene duplications in a strain infecting diverse plant orders (Normand et al. 2007). Collectively, these observations raise the possibility that gene duplications and gene family expansions are common processes facilitating adaptation to diverse ecological niches across various organisms. Therefore, it would be reasonable to assume that the gene duplication of GRs in the *V. cardui* lineage are associated with adaptation to diverse host plants. However, a more comprehensive study including multiple generalist lineages will be necessary to investigate whether the pattern we observed is peculiar to generalist lineages or specific to *V. cardui*.

### Taxon-Specific GR Subfamilies and Perception of Plant Secondary Metabolites

The phylogenetic analysis identified two taxon-specific GR subfamilies, LS and BS (fig. 2). These subfamilies were characterized by frequent lineage-specific gene gains and losses, including genes duplicated in the recent *V. cardui* lineage (fig. 2), and higher evolutionary rates compared with sugar and CO₂ clades (fig. 3). Based on these results, we assume that genes in LS and BS are involved in the perception of plant secondary metabolites by nymphalid butterflies. In *Drosophila*, the GR subfamily responding to bitter tastants, such as plant-derived secondary metabolites, evolved faster than sugar and CO₂ subfamilies (McBride et al. 2007; Weiss et al. 2011; Ling et al. 2014; Delventhal and Carlson 2016; supplementary fig. 4 and supplementary table 10, Supplementary Material online), which resembles the estimation for LS and BS in our data. Moreover, more than half of lost genes during host specialization in *Drosophila* belonged to the putative bitter receptor clade (McBride et al. 2007; supplementary table 10, Supplementary Material online), which is consistent with the frequent turnover of GR gene repertoires in LS/BS. As secondary metabolite repertoires on plants are likely to be quite variable at both intra- and inter-species scales, divergent evolution of GRs in LS/BS in terms of sequences and gene repertoires might reflect their role as receptors for secondary metabolites. The fact that GRs for synephrine receptor in *P. xuthus* (Ozaki et al. 2011) and those
expressed exclusively in female legs in *H. melpomene* (Briscoe et al. 2013) were included in BS also imply that the subfamily plays some roles in host selection. Although GRs in LS do not have empirical knowledge to support their functions, three GRs of *H. armigera*, which could be classified in LS in our gene phylogeny, responded to extracts of cotton leaves, suggesting their functions as bitter taste receptors (Xu et al. 2016). Overall, these facts imply that GRs for secondary metabolite receptors of nymphalid butterflies are most likely to exist in the taxon-specific subfamilies. We acknowledge that we cannot ultimately verify their roles without functional analysis, thus candidate bitter GRs should be deorphanized in the following studies.

Expansion of Detectable Secondary Metabolites May Be an Adaptation to a Wider Host Plant Range

Our findings together suggest that frequent gene duplications in GRs, which might be responsible for secondary metabolite detection, were associated with host range expansion in Nymphalini. Given the variation of ligand affinities among GRs, the increase in the number of GRs is likely to reflect the repertoire expansion of detectable compounds during host plant selection by ovipositing females. For herbivorous insects, especially butterflies, plant secondary metabolites are classified either as a stimulant or deterrent, which respectively induces or prevents oviposition (Renwick and Chew 1994). Thus, the increase in GRs can be interpreted as the repertoire expansion of detectable stimulants or deterrents, which is possibly an adaptation to diverse host plants.

If the increase in GRs represents an expansion of detectable deterrents, duplication in GRs would not be the direct cause of the host range expansion because adding new deterrents into the repertoire would prevent colonization of novel plant taxa. However, once the host range is expanded by other mechanisms, insects may lay eggs on less suitable species for newly included host plant taxa, because they may not sense the compounds that represent toxicity of the new taxa. In this situation, the increase in GRs would be favored in generalists because it can enhance the accuracy of host selection by expanding repertoires of detectable deterrents. In contrast, GRs would not increase as long as the lineage remains specialized on specific plant taxa. This concept is consistent with an explanation for herbivorous vertebrates tending to have larger numbers of bitter taste receptor genes: recognition of various compounds would be adaptive for accurate discrimination of better plants for food (Li and Zhang 2014).

On the other hand, it is also possible that the increase in GRs represents an expansion of detectable oviposition stimuli. When butterflies include new compounds into their stimulant repertoire, they may start to recognize some plants that were previously avoided as oviposition sites. Thus, in this case, duplication of GRs can trigger host range expansion. Under this assumption, the number of GRs would be increased under selection in species or populations that favor a more extensive host plant range, whereas the number would be stable as long as specialized hosts are favored and maintained.

We are unable to determine which scenario is more plausible at this point. In fact, the two scenarios of adaptation may have occurred simultaneously in the course of host range expansion. To understand the evolutionary relationships between the number of GRs and host range expansion in detail, we have to answer more questions, such as the timings for duplication and fixation of GRs, ligand affinities and behavioral influences of each receptor, and distribution of ligands on candidate host plant taxa in nature.

Conclusion

Although a substantial number of studies have focused on oviposition behavior of butterflies, few studies revealed mechanisms of evolutionary transition between specialists and generalists. Our analysis of nymphalid butterflies, including the generalist *V. cardui*, showed that rapid GR gene duplications have occurred during host range expansion and that there were two taxon-specific GR subfamilies that may be related to secondary metabolite detection. Together, the results imply that the expansion of detectable secondary metabolites during host selection was an adaptation to various host plants by generalist butterflies. These findings would establish the foundation for understanding the evolution of plant–insect interactions, and how it has facilitated species diversification.

Supplementary Material

Supplementary data are available at Genome Biology and Evolution online.

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