Chance and predictability in evolution: the genomic basis of convergent dietary specializations in an adaptive radiation

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Abstract

The coexistence of multiple eco-phenotypes in independently assembled communities makes island adaptive radiations the ideal framework to test convergence and parallelism in evolution. In the radiation of the spider genus *Dysdera* in the Canary Islands, species diversification occurs concomitant with repeated events of trophic specialization. These dietary shifts, to feed primarily on woodlice, are accompanied by modifications in morphology (mostly in the mouthparts), behaviour and nutritional physiology. To gain insight into the molecular basis of this adaptive radiation, we performed a comprehensive comparative transcriptome analysis of five Canary Island *Dysdera* endemics representing two evolutionary and geographically independent events of dietary specialization. After controlling for the potential confounding effects of hemiplasy, our differential gene expression and selective constraint analyses identified a number of genetic changes that could be associated with the repeated adaptations to specialized diet of woodlice, including some related to heavy metal detoxification and homeostasis, the metabolism of some important nutrients and venom toxins. Our results shed light on the genomic basis of an extraordinary case of dietary shift convergence associated with species diversification. We uncovered putative molecular substrates of convergent evolutionary changes at different hierarchical levels, including specific genes, genes with equivalent functions, and even particular amino acid positions. This study improves our knowledge of rapid adaptive radiations and provides new insights into the predictability of evolution.

Keywords: Oceanic islands, Spiders, Diet specialization, Comparative transcriptomics, Differential gene expression, Positive selection, Heavy metals, Toxins, Phenotypic convergence

Introduction

The current limited knowledge of the evolutionary mechanisms underlying diversification compromises our ability to manage and conserve biodiversity (Mergeay & Santamaria, 2012). Evolutionary biology provides a unifying conceptual framework to successfully identify key diversification drivers through the study of molecular variation. As many other fields, evolutionary biology has fully entered the genomics era, which opens up the possibility of tackling longstanding questions regarding biodiversity in a more fruitful way and at a lower cost (Losos et al., 2013). Although often seen as a gradual process that requires the action of different evolutionary forces acting steadily over long periods of time (Coyne & Orr, 2004), speciation can be very rapid under unstable environmental and ecological conditions. In fact, one of the most promising approaches to disclose the relative impact of these driving forces is the study of species radiations in nature, i.e., the rapid appearance of a high number of species from a single common ancestor (Schluter, 2000). In adaptive radiations, such as the classic examples of Darwin's finches (Almén et al., 2016) and the cichlids in the great lakes of Eastern Africa (Henning & Meyer, 2014), significant morphological differences appear over short periods of time despite the low levels of genetic divergence accumulated at the genomic level. Nevertheless, the relative role of natural selection and of other non-adaptive forces in such relevant evolutionary processes is a matter of scientific debate (Muschick, Indermaur, & Salzburger, 2012).

Oceanic islands are considered natural laboratories for studying evolution. The entire biota of these islands is derived from a few initial colonization events followed by local diversification, which generates high levels of endemism and ecomorphological differentiation (MacArthur & Wilson, 1967; Mayr, 1942; Whittaker & Fernández-Palacios, 2007). Thus, the biota of oceanic islands can be interpreted as the result of successful

independent evolutionary experiments starting with a single or multiple colonization events from the continent (Emerson, 2002). The comparative analysis of such independent events and the subsequent island radiation (both within and between islands) in different archipelagos provides new insights into the general evolutionary process generating biological diversity (Gillespie & Roderick, 2002; Losos & Ricklefs, 2009). Such approximation has been successfully applied in a number of studies on oceanic islands (Losos, Jackman, Larson, Queiroz, & Rodriguez-Schettino, 1998; Stroud & Losos, 2016), such as Hawaii (Gillespie, 2004), the Galapagos (Grant & Grant, 2008) and the Canary Islands and Madeira archipelagos (Juan, Emerson, Oromí, & Hewitt, 2000; Machado, Rodríguez-Expósito, López, & Hernández, 2017), where explicit hypotheses on the evolutionary processes underlying radiations have been tested.

The radiation of the genus *Dysdera* Latreille, 1804 (Araneae: Dysderidae) in the Canary Islands is one of the most spectacular examples of island species diversification within spiders (Arnedo, 2001; Arnedo, Oromí, Múrria, Macías-Hernández, & Ribera, 2007). As many as 47 endemic species of this species-rich Mediterranean genus (approximately 250 species) have been reported in the Canary Islands (Macías-Hernández, López, Roca-Cusachs, Oromí, & Arnedo, 2016; World Spider Catalog, 2019). The spiders of the genus *Dysdera* are active nocturnal hunters that spend the daytime in silk retreats and are usually found under stones, dead logs or leaf litter or even living in caves (Arnedo et al., 2007). This genus stands out among spiders in having evolved trophic specialization; i.e., several species have been shown to feed preferably (facultatively or even obligatorily) on terrestrial woodlice (Crustacea: Isopoda) (Řezáč & Pekár, 2007; Řezáč, Pekár, & Lubin, 2008), a prey rejected by most generalist predators (Pekár, Líznarová, & Řezáč, 2016). Available evidence suggests that prey specialization (i.e., stenophagy) has appeared several times, both on the continent

and on the islands. Interestingly, the morphology of mouth parts predicts both dietary preferences and capture strategy (chelicerae used as pincers, forks or keys) and the frequency of captures among the specialists (Řezáč et al., 2008). All cheliceral types observed in continental species have also evolved repeatedly in the Canary Islands, suggesting that prey segregation is a major driving force of the spectacular diversification of the genus on the islands (Arnedo et al., 2007). Woodlice are a difficult prey for other arthropods because of their morphological, chemical and behavioural defences (Gorvett, 1956; Sutton, 1980). These defences comprise dorsally protective armour, gland secretions producing repulsive odours, indigestibility to many predators, and behavioural patterns such as nocturnal activity, rolling into a ball or adhering to surfaces when threatened (Schmalfuss, 1984; Sutton, 1980). In addition, these organisms accumulate high concentrations of heavy metals from the soil, making them even more toxic to predators (Drobne, 1997). Consequently, woodlice are rarely eaten by generalist predators. Within arthropods, only spiders and ants have developed specialized strategies to feed on this prey (Dejean, 1997; Pekár et al., 2016). Nevertheless, despite all this morphological and experimental evidence, the genetic basis of this remarkable adaptation is completely unknown.

Moreover, the study of the molecular basis of such an extraordinary phenotypic convergence offers an opportunity to address the question of predictability and repeatability of the evolutionary process. Given that it is not possible to rerun the tape of evolution, the study of parallel evolutionary outcomes in different scenarios provides a fairly good framework to ascertain both to what extent similar molecular solutions has been exploited repeatedly, and which aspects are predictable at different hierarchical levels (i.e., at the nucleotide, gene, pathway or function level). Among *Dysdera* spiders, the specialized woodlice eaters (i.e., oniscophagous species) possess, in addition to the morphological modifications of chelicera,

important behavioural and nutritional adaptations to feed on isopods (Hopkin & Martin, 1985; Řezáč & Pekár, 2007; Toft & Macías-Hernández, 2017). With the aim of understanding the genetic basis of these specific adaptations and to shed some light on the longstanding debate of how predictable is molecular evolution, we designed a case study that included adult individuals from two pairs of recently diverged endemic specialist-generalist species from the Canary Islands, likely representing two phylogenetically and geographically independent dietary shifts from a generalist ancestor. Our survey included the GV pair: *Dysdera gomerensis* Strand, 1911 (El Hierro) and. *D. verneaui* Simon, 1883 (Tenerife), the TB pair: *D. tilosensis* Wunderlich, 1992 and. *D. bandamae* Schmidt, 1973 (Gran Canaria), and a third generalist endemic species external to both pairs: *D. silvatica* (La Gomera) (Arnedo pers. Comm; Macías-Hernández, Oromí, & Arnedo, 2008; Vizueta et al., 2017), which was used as an outgroup (Figure 1). We compared the transcriptome profiles and the selective constraint patterns between specialists and generalists to identify the genomic regions responsible for the rapid dietary adaptation of *Dysdera* species in the Canary Islands. We studied transcriptomic data from adult individuals, we were able to detect putative adaptive changes associated with food detection and assimilation, including its digestive and metabolic aspects. True homoplasy can arise by evolving the same (or similar) trait from either a non-shared common ancestor (convergent evolution) or a shared ancestor but through evolutionarily independent events (parallel evolution). Here, we will refer to both cases with the general term of "convergence". We aimed to detect those evolutionary changes required to explain a repeated character state in the two specialist lineages, either a gene expression profile or a selective constraint pattern, matching phenotypic convergence. Nevertheless, both incomplete lineage sorting of (ILS; Maddison, 1997) and species hybridization can produce fundamental discordances between gene trees and the species tree, a phenomenon commonly referred to as "hemiplasy" (Avise & Robinson, 2008), giving rise to the illusion of homoplasy

147 and the erroneous inference of convergence (Mendes, Hahn, & Hahn, 2016; Wu, Kostyun,
148 Hahn, & Moyle, 2018).
149
150 Here, and after controlling for the potential confounding effects of hemiplasy, we identified
151 clear signals of homoplasy at different hierarchical levels likely attributable to adaptive
152 convergence in specialist species. Noticeably, we even find signals of this adaptive process at
153 the amino acid level. The repeated changes matching phenotypic convergence found in this
154 study mostly affected genes and gene functions associated with the strategy of detoxifying
155 heavy metals (and perhaps other toxic substances) accumulated by woodlice, to the enhanced
156 assimilation of some nutrients and, to a lesser extent, to venom composition.
157

158 # Material and Methods

159 ## Study design and sample materials

160 Our study design included two pairs of phylogenetically related *Dysdera* species endemic
161 from the Canary Islands. Each pair of close relatives was composed of a generalist and a
162 specialist (stenophagous) species regarding their diet and shared a generalist ancestor, which
163 implies that at least two specialization events occurred independently during the divergence
164 of these four species, one on each species pair (Figure 1). Both the phylogenomic analysis
165 performed here and recent multi-locus based phylogenies including other endemic species of
166 this genus (Arnedo et al. unpublished results) indicate that *D. gomerensis* and *D. verneaui* are
167 true sister taxa, while *D. tilosensis* and *D. bandamae* are very closely related, although it is
168 difficult to know if they are each other closest relatives. Similarly, the ancestral state
169 reconstruction supports that the ancestor of the complete Canarian radiation was a generalist,
170 while *D. tilosensis* is a derived specialist from a generalist ancestor. For the case of *D.*
171 *gomerensis* this is much more difficult to establish because of the phylogenetic uncertainty,
172 probably due to a very rapid radiation of these species group. In any case, this rapid radiation
173 however makes that most candidate changes in the *D. gomerensis* lineage (see below), would
174 be adaptations to stenophagy, independently of whether the ancestor was a complete
175 generalist, or just a facultative intermediate.
176
177 The two specialists species of our study show modifications in their mouthparts that have
178 been associated with a preference for using isopods as a prey (Řezáč et al., 2008; Macías-
179 Hernández et al, in prep) (see Figure 1). We collected 16 individuals of *Dysdera tilosensis*
180 (10 males and 6 females) and 14 individuals of *D. bandamae* (5 males and 9 females) in Gran
181 Canaria, and 12 males of *D. verneaui* in Tenerife and 15 females of *D. gomerensis* in El
182 Hierro (Table S1). We also included in the analysis a fifth Canary Island endemic *Dysdera*

183 species, the generalist *D. silvatica*, as an outgroup and to polarize the evolutionary changes in
184 internal branches (Vizueta et al., 2017) (Figure 1).
185
186 **Transcriptomic analysis**
187 For each species, we sequenced the transcripts from the palps (*PALP*), the first pair of legs
188 (*LEG#1*), all other legs (*LEG#234*), and the rest of the body (*REST*), separately in four
189 different RNAseq experiments. We applied this strategy to maximize the detection of low
190 expressed genes, especially chemosensory gene family members in spider appendices (see
191 Vizueta et al., 2017 and Frías-López et al., 2015; Supplementary Methods). Specimens were
192 starved for two weeks at the laboratory and posteriorly fixed in liquid nitrogen and stored at
193 −80 °C until further processing. From the total RNA, we sequenced the transcriptomes in the
194 Illumina HiSeq 4000 platform using pair-end libraries (100-bp reads; Table S1). A detailed
195 description of raw data pre-processing, transcriptome assembly and functional annotation of
196 the transcripts from the four species is available in Supplementary Methods.
197
198 **Species-tree, gene-tree discordance, and risk of hemiplasy**
199 We identified all groups of homologous genes that share at least one member in the ancestor
200 of the five *Dysdera* species (i.e., orthology groups) using OrthoMCL with default parameters
201 (Li, Stoeckert, & Roos, 2003). We further separated single-copy orthologs from multigene
202 families. Since at the moment of starting this work, all published phylogenetic analyses
203 including the studied species were based on few genes (Arnedo, 2001; Arnedo et al., 2007),
204 we performed a more comprehensive phylogenomic analysis using all single copy orthologs
205 across the five Canarian *Dysdera* species plus *D. crocata* Koch, 1839 (the phylogenetically
206 closest continental species of this genus with available transcriptome data; Fernández,
207 Hormiga, & Giribet, 2014) (Figure 2). Only complete or nearly complete transcripts free of

premature stop codons were included in the analysis. The multiple sequence alignments (MSA) of the CDS of each orthology group were generated with the program T-Coffee (Notredame, Higgins, & Heringa, 2000) and further concatenated in a single MSA using in house Perl scripts. We set the GTRGAMMA substitution model in a partitioned scheme to obtain the maximum likelihood (ML) tree in the software RAxML (Stamatakis, 2014). Model parameters were estimated independently for each single-copy ortholog and node support was obtained after 500 bootstrap replicates.

We approximated the divergence times between the five Canarian *Dysdera* species by fitting the data from single copy orthologs to the unrooted tree topology of the ML tree after excluding *D. crocata*. We set the same substitution model and partition scheme than in the previous RAxML analysis. We used the penalized likelihood method of Sanderson (2002), implemented in the program r8s v1.80, to generate the ultrametric tree and to estimate node ages (Sanderson, 2003). We set a calibration point in the node representing the split of the *D. silvatica* lineage from the rest of lineages (3.4-7.8 Mya range; Macías-Hernández, Bidegaray-Batista, Emerson, Oromí, & Arnedo, 2013).

We also inferred a species tree that incorporates gene-tree uncertainty using ASTRAL (Zhang, Rabiee, Sayyari, & Mirarab, 2018). For that, we first estimated the ML tree of each individual MSA (i.e., a gene tree for each single-copy ortholog) with RAxML (setting the GTRGAMMA substitution model and calculating node support with 1000 bootstrap replicates). Moreover, we estimated the Hemiplasy Risk Factor (HRF) along the phylogeny using the PePo package (Guerrero & Hahn, 2018). For the analysis, we used the species tree inferred with ASTRAL (with branch lengths in $2N_e$ generation units), a very approximate estimate of the population scaled mutation rate in *D. silvatica* ($\theta = 0.011$; estimate obtained

from a short read alignment to the first genome draft of this species; Sánchez-Herrero et al., 2019), a generation time of 1.5 years, and six different effective population sizes, N_e (10^3, 5 x 10^3, 10^4, 5 x 10^4, 10^5 and 10^6). Finally, all candidate genes exhibiting resolved discordant topologies (i.e., with bootstrap support ≥ 75% in at least one node producing discordance with the species tree) were excluded for the downstream functional prediction analyses and their interpretation. Finally, we used the D_{FOIL} statistic (Pease & Hahn, 2015) to test for introgression between the specialist lineages in presence of ILS, using both *D. silvatica* or *D. crocata* as outgoups.

Differential expression analyses

Differential expression (DE) analyses were performed separately in each generalist-specialist pair (GV and TB pairs; see Figure 1; Supplementary Methods). Raw reads of the RNAseq from each species and body part were mapped back to their own reference CDS and to the CDS of the other species in the pair by using BOWTIE2 version 2.2.3 (Langmead & Salzberg, 2012). Read counts and TMM-normalized FPKMs (i.e., trimmed mean of log-expression ratios-normalized fragments per kb of exon per million reads mapped) were estimated for single-copy genes and multigene families using RSEM 1.2.19 software (Li & Dewey, 2011). To test for genes showing DE between specialists and generalist species, we calculated the negative binomial dispersion of read counts across species pairs of a set of housekeeping (HK) genes with EdgeR version 3.18.1 (Robinson, McCarthy, & Smyth, 2010). We used this dispersion to conduct the DE analysis between specialist and generalist species. We merged all body parts (within a species) to homogenize the differences in the number of REST samples between species pairs. To avoid type I and II errors associated to this merging, especially when gene expression is higher in *REST* relative to legs (both *LEG#1* and *LEG#234*) and *PALP*, we used total read counts from all samples normalized for each library

size to perform differential expression analyses. The *P*-values of these analyses (one per gene) were corrected for the false discovery rate (Benjamini & Hochberg, 1995) (FDR). We considered that a gene is differentially expressed between two species when expression levels are significantly different with a FDR < 0.05.

Selective constraints analyses

We used the adaptive Branch-Site Random Effects Likelihood (aBSREL) model implemented in the HyPhy package (Pond, Frost, & Muse, 2005; Smith et al., 2015) to test if positive selection has occurred repeatedly in the same gene in specialist lineages. This method is based on the parameter ω (the ratio of nonsynonymous (d_N) to synonymous (d_S) substitution rates, $\omega = d_N/d_S$) and allows fitting an optimal number of ω classes to codon sequence alignments of single-copy orthologs in each branch of the phylogeny (Figure 2; Supplementary Methods). Positive selection is inferred when a gene shows codons fitting a class with $\omega > 1$ in a particular lineage. We also tested for relaxation or intensification of the strength of natural selection in these single copy orthologues in specialist lineages using the RELAX framework in HyPhy (Wertheim, Murrell, Smith, Kosakovsky Pond, & Scheffler, 2015). Besides, we applied the Mixed Effects Model of Evolution (MEME) implemented in the HyPhy package (Murrell et al., 2012) to identify individual sites evolving under episodic positive selection (in one or more lineages) in the set of candidates from PCOC analysis (see below). Both methods are based on the same principle of aBSREL of fitting different probabilistic models of the ω parameter distribution, and also inferred positive selection when $\omega > 1$. Finally, we applied the aBSREL model to test for episodic positive selection acting on gene families in specialist lineages. In this case, we used the same workflow as for the single copy orthologs but applying the FastTree program (Price, Dehal, & Arkin, 2010) to approximate a ML tree of each family.

283

284 **Convergent amino acid evolution**

285 To detect convergent amino acid evolution in specialist lineages, we aligned the amino acid
286 sequences of the PS candidates using the software PRANK and applied the method PCOC
287 (Rey, Guéguen, Sémon, & Boussau, 2018) (Profile Change with One Change), a recently
288 developed approach to identify convergent shifts in the amino acid substitution rate across a
289 phylogeny, to each individual MSA. Moreover, we used computer simulations to test the
290 performance of PCOC method with our empirical data. We applied the same species tree,
291 average sequence length and model parameters set in the PCOC analysis of the observed data
292 to simulate sequences both with convergent (2% of sites undergoing convergent amino acid
293 substitutions) and without convergent changes (Rey et al., 2018). Using these simulated
294 sequences, we estimated the false discovery rate (FDR; using simulations without
295 convergence) and true positive rate (TPR; using simulations with convergent amino acid
296 substitutions) associated with this analysis.

297

298 **GO enrichment**

299 We used R and GOstats (Falcon & Gentleman, 2007) to carry out the gene ontology (GO)
300 enrichment analysis and REVIGO (Supek, Bošnjak, Škunca, & Šmuc, 2011) to generate a
301 graphical representation of the results. We also used Blast2GO suite (Conesa et al., 2005) to
302 identify KEGG pathways enriched in the list of candidates (Kanehisa & Goto, 2000).
303 Hypergeometric tests were performed with dhyper function of the R package STATS.

304

Results

We constructed 16 RNA-seq datasets (four different body parts in four species) to obtain four new complete *Dysdera* transcriptomes (Table S1). As expected, both the number of species-specific transcripts (from 170,846 to 347,878) and the number of functionally annotated genes differed between species (Table 1), but the transcriptome completeness, measured as the number and integrity of CEG genes, was quite similar (Table S2). Only 30% of the transcripts encoded protein-coding genes; the rest corresponded to either non-coding transcripts or assembly artefacts (Table 1). Furthermore, ~35% of the predicted proteins showed no significant sequence similarity or conserved profiles with known arthropod genes (i.e., putative orphan genes of the *Dysdera* lineage). Among the annotated proteins, most were chelicerate specific, and ~66% of the top BLAST hits matched spider sequences (Figure S1).

We identified a total of 13,947 orthologous groups across the five Canarian *Dysdera* species, of which 7,958 were free of premature stop codons, and 4,539 showed complete sequences in all species (Figure 2). The number of single-copy orthologues across the five species was 9,473, a number that increased to 19,497 in the GV pair and 24,212 in the TB pair (Table S3). The maximum likelihood (ML) tree that included *D. crocata* (2,472 genes; 2,926,723 bases) confirmed the expected phylogenetic relationships (Figure 1), i.e., that *D. silvatica* is sister to the two generalist/specialist sister lineages (GV and TB). We estimated that *D. gomerensis* and *D. verneaui* diverged approximately ∿4.1 Mya, whereas the split between *D. tilosensis* and *D. bandamae* occurred ∿3.1 Mya; the age of the common ancestor of these four lineages dates to ∿4.5 Mya (analysis based on 4,539 genes; Figure 1). These estimates are similar to those obtained in Macías-Hernández et al., (2013).

These very recent divergence times, especially the short internal branch lengths, indicated that hemiplasy might represent an important confounding factor in our inferences of convergent evolution. Indeed, although the species tree estimated with ASTRAL had the same fully supported topology (the local posterior support for each branch was 1) than as the ML tree based on the concatenated MSA, the final normalized quartet score of this species tree (0.65) uncover a high gene tree conflict in our data set. The risk of hemiplasy (HRF) estimated along the species tree obtained with ASTRAL, varied according to the effective population sizes and the examined branch (Figure 3), being small for $N_e \leq 10^4$, high in branches A and C for $N_e \geq 10^5$, and extremely high in all branches for $N_e \geq 10^6$. Given the high fraction of discordant gene trees observed in our data (5,275 out of 7,784 gene trees; 3,666 with high bootstrap support ≥ 0.75 in at least one discordant node) together with HRF estimates, the surveyed species (and their ancestors) would have intermediate to high effective population sizes, in a range of $10^4 < N_e \leq 10^6$. Although only a small fraction of these inconsistencies might really affect our inferences of homoplasy (see discussion), we specifically considered this confounding factor in our study. In contrast, we did not detect the characteristic hallmark of gene flow between extant specialist lineages in the D_{FOIL} analysis of transcripts, neither by analyzing all transcripts separately nor concatenating them in different gene groups (i.e., all transcripts, all candidates, only gene expression, or only positive selection candidates; results not shown; see below for the precise definition of each type of candidate).

Gene expression changes matching phenotypic convergence: individual gene level

Despite the sex-ratio bias of the studied samples (Table S1), the PCA analysis of the eight *REST* samples of the specialist *D. tilosensis* sequenced separately (four males and four females), showed no evidence of sex-specific expression (Figure S2), which is in agreement

with the absence of morphological dimorphism between sexes reported for the Eastern Canarian clade of this genus (Macías-Hernández et al., 2008). We found 774 (out of 19,497) and 1,044 (out of 24,212) genes showing differential expression between specialists and generalist species in the GV and TB pairs, respectively (Figure S3; Table S4). Remarkably, 147 genes (out of 193) had patterns of gene expression matching phenotypic convergence, i.e., the expression profiles had the same trend in both species' pairs with the two specialists significantly under- or overexpressed (hereafter referred to as Matching Gene Expression "MGE" candidates); however, in three cases the tree showed discordant genealogies supported by the entire transcript sequence. The final number of MGE candidates (144 genes) is much higher than that expected by a neutral model of gene expression evolution, both when considering all differentially expressed genes (hypergeometric test; $P = 1.3x10^{-67}$) and separating genes over- or underexpressed in specialist lineages ($P = 2.3x10^{-14}$ and $P = 4.2x10^{-121}$, respectively; hypergeometric test). The proportion of genes significantly underexpressed in specialists was higher both in the two species pairs considered separately (68% in GV and 61% in TB) and, to a much greater extent, across the 144 shared DE candidate genes (114 genes; 79%) (Figure 4; Table S4). All MGE candidates except two functionally uncharacterized proteins (OG9619 and OG15050 in *PALP*) and one phosphatase (OG1641 in *LEGS*), were predominantly expressed in *REST*, (Figure 4; Figure S3), and none of them show DE between males and females of *D. tilosensis* in this body part (results not shown). All these findings indicate that DE analyses are reflecting real differences between specialist and generalist species, and not sex or body part-specific features. Yet, we cannot completely rule out that some of the uncovered candidates ~~was~~ were ~~a~~ false positives, so they should be considered as promising candidates to be further validated.

Within the biological processes significantly overrepresented (Figure 5a) among MGE candidates, we identified genes involved in the homeostasis of metal ions; catabolism of amino acids, sugars and chitin and activities of enzymes such as phosphatase and hydrolase. The separate analysis according to the direction of gene expression change showed that the 114 MGE candidates downregulated in specialists are significantly enriched in assembly and organization of chromatin, cytoskeleton and other cellular structures (such as the organelles), potential regulation of developmental processes through the smoothened pathway, cell morphogenesis and growth processes, and catabolism of sugars and amino acids. In contrast, the 30 MGE candidates upregulated in specialists are significantly enriched in GO terms associated to the metabolism of steroids, lipids and dicarboxylic acid, the activities of phosphatases and hydrolase, the membrane transport of different substances, and responses to various external stimuli including cellular response to oxidative stress. Other interesting but not GO-enriched functions of the MGE candidates include iron ion binding (a predicted cytochrome P450 protein overexpressed in specialist spiders) and zinc ion binding (mostly represented by various putative zinc finger-containing proteins; Table S4). Furthermore, we also found two putative venom toxins among the 144 MGE candidates, one of which encodes a protein similar to the α-latrocrustatoxin (underexpressed in specialists), while the other is an U32-aranetoxin-Av1a overexpressed in specialists (see Figure S4 and Table S4 for a more detailed functional description of the MGE candidates, including significantly enriched molecular functions).

Our analysis also detected 21 genes specifically expressed in specialists (i.e., with no detectable expression in generalists; referred to as Matching Specialist-specific Expression "MSE" candidates) (Figure 2). Fifteen of these MSE candidates encode proteins with no significant sequence similarity with any entry in the searched databases; the other six cases,

405 which were not enriched in any GO term, encode catalytic activities, such as hydrolases and
406 peptidases, or are associated with zinc ion-binding proteins, likely involved in the regulation
407 of gene expression (Table S4).
408
409 The highly fragmented nature of the transcripts encoding members of the major
410 chemosensory gene families (Vizueta et al., 2018) prevented the credible assignation of many
411 orthogroups and, therefore, a reliable DE analysis comparing specialists and generalists.
412 Besides, for the few orthogroups that could be assigned, we did not find any concordant DE
413 pattern in specialists. The same negative results were obtained for the other orthogroups that
414 showed DE in the chemosensory appendages (*PALP* and *LEG#1* and *LEG#234*) in the study
415 of Vizueta et al., (2017).
416
417 **Gene expression changes matching phenotypic convergence: gene function level**
418 Apart from the 144 MGE candidates, the group of genes with DE only in one species pair,
419 627 in GV pair and 897 in TB pair, respectively, also shared a significant number of enriched
420 GO terms (70 terms; hypergeometric test, $P = 4.7 \times 10^{-11}$ for all DE genes; $P = 2.2 \times 10^{-23}$ and P
421 $= 1.3 \times 10^{-2}$ for under- and overexpressed genes, respectively). Remarkably, some of these GO
422 terms are the same as those overrepresented among the MGE candidates. For the genes
423 underexpressed in specialists, these included chromatin assembly, the organization of cellular
424 components, such as the cytoskeleton or organelles, and cell growth. Other additional
425 functions, such as phosphate metabolism regulation and the apoptotic process involved in
426 morphogenesis, are also shared among these genes. For the genes overexpressed in
427 specialists, the enriched functions shared between species pairs include lipid catabolism,
428 oxidation-reduction process and response to antibiotics (Figure S4 and Table S4).
429

Among the orthogroups with DE only in one species pair but with equivalent functions, we found genes involved in detoxification processes and genes encoding various members of the cytochrome P450 family (most of them overexpressed in specialists, seven and nine different copies in the GV and TB pairs, respectively) or proteins with esterase activity (seven and six of these enzymes in the GV and TB pairs, respectively). Additionally, we found 29 putative venom toxin-encoding genes in the GV pair (eight overexpressed in G) and 34 in the TB pair (26 overexpressed in T). Interestingly, although the encoding genes differed between the two specialists, they had very similar predicted functions, such as astacin-like metalloprotease toxin precursors or aranetoxin-Av1a and latrotoxins, among others (Table S4).

Positive selection matching phenotypic convergence: individual gene level

We applied the aBSREL model to estimate the distribution of ω values of all single-copy orthologues with complete sequences and without premature stop codons (7,784 genes; Figure 2; Table S3). This genome-wide analysis uncovered opposite trends between GV and TB pairs; while the overall selective constraints appear to have been relaxed in the *D. tilosensis* lineage, they intensified in the *D. gomerensis* branch (Figure S5). Nevertheless, the analysis of individual genes identified nine genes with significant differences in the selective constraint values shared between the two specialists (or the two generalists) (RELAX framework analysis, FDR of 0.2; Table S5; referred as Matching Functional Constraint "MFC" candidates). Six of these candidates showed the relaxation hallmark in specialists, while the other three showed a significant increase in the selective constraint. We found some overrepresented biological functions among MFC candidates, such as carbohydrate metabolism and homeostasis, neuropeptide signaling, tRNA modification and pyridine metabolism (Figure S4). When we considered not enriched GO terms, the genes with increased functional constrains in specialists encode proteins similar to the membrane

glycoprotein LIG-1, a neuropeptide receptor-like protein, and zinc finger proteins while the genes that have relaxed most in specialist's species encode two zinc finger-like proteins and a hexokinase.

We identified 297 genes with significant evidence of positive selection in specialist lineages, 169 in *D. gomerensis*, 150 in *D. tilosensis* and, remarkably, 22 cases in which positive selection was inferred in both dietary specialists (Figure 2; Table S6; referred to as Matching Positive Selections "MPS" candidates). After excluding five coding regions with discordant genealogies supported by the entire transcript sequence, the number of MPS candidates (17) is clearly greater than that expected by chance (across the 297 genes showing positive selection in specialists; hypergeometric test; $P = 1.5x10^{-8}$). These genes are enriched in biological processes such as germ cell migration and cell death, cell junction assembly and organization, regulation of the immune response or iron ion homeostasis (Figure 5; Figure S4). Interestingly, one of these genes with endopeptidase inhibitor activity encodes a protein with sequence similarity to U24-ctenitoxin-Pn1a, a possible venom toxin related to cysteine proteinase inhibitors.

The PCOC method (Rey et al., 2018) identified convergent shifts in amino acid preferences in 14 out of the 17 MPS candidates (FDR = 0.03%; TPR = 99.7%; Figure 6; Table S6; Figure S6). Furthermore, in five cases, the subsequent MEME analysis indicated that some of the amino acid sites involved in these convergent shifts have also evolved by positive selection (8 amino acid sites; Figure 6). The target genes include i) the U24-ctenitoxin-Pn1a candidate toxin (OG6752 orthogroup; 6 amino acid changes); ii) OG7181, a transcript encoding a protein similar to tectonin (10 amino acid changes, 3 of them under); iii) OG9641, a transcript encoding a protein involved in response to oxidative stress (3 amino acid changes,

one of them also detected with MEME); iv) OG11255, a gene that encodes a product similar to a mannose receptor (5 amino acid changes, 2 of them also detected with MEME); v) OG13286, a protein likely encoding a sodium channel (1 amino acid change, also detected with MEME); and vi) OG16682, a hydrolase involved in nitrogen compound metabolism (4 amino acid changes, one of them detected with MEME). The analysis also inferred some amino acid substitutions responsible of a convergent shift of preferences in specialists but without evidence of positive selection in OG9529, a putative dehydrogenase and oxidoreductase (4 amino acids) (Figure S6).

Positive selection matching phenotypic convergence: gene function level

Although the group of genes under positive selection in only one of the two specialists (147 in GV pair and 138 in TB pair, respectively) did not share more significantly enriched GO terms than expected by change (only three shared GO were enriched in both pairs; hypergeometric test; $P = 0.19$), the number of total GO terms shared by these two groups is greater than expected ($P = 5.3 \times 10^{-75}$ based on the hypergeometric distribution). Among shared GO terms, we found processes and functions such as chitin metabolism (including proteolysis activity), lipid metabolism, metal ion binding (zinc in both pairs, copper in *D. gomerensis* and iron in *D. tilosensis*), and hydrolase and oxidoreductase activities (Figure S4). In addition, we also detected the signature of positive selection in six genes encoding putative venom toxins: four in *D. gomerensis* and two in *D. tilosensis* (Table S6).

The gene family analysis also uncovered the hallmark of positive selection in five gene families affecting both specialist lineages (Figure 2; Table S6). One family (the OG3133 orthologous group), which included sequences without any functional annotation, also showed copy number variation in the two specialists (2 and 3 copies in *D. gomerensis* and *D.*

505 *tilosensis*, respectively, compared to one in the generalist species). The other four gene
506 families encoded proteins with possible functions in chitin metabolism and sequences similar
507 to carbohydrate and zinc ion-binding proteins, hydrolases and other enzymes with catalytic
508 activity. Again, we found a gene family encoding putative venom components (in this case,
509 with no characterized target) among positively selected gene families.
510

511 ## Discussion

512 The evolution of stenophagy, dietary specialization from a generalist ancestor, most likely
513 involves gene regulatory changes, amino acid replacements in proteins, and/or even copy
514 number variation in gene families. Here, we focused our analysis on the first two issues since
515 comparative transcriptomics based on *de novo* assemblies prevents accurate estimation of
516 changes in gene expression and gains and losses in gene family members. Our approach
517 allows detecting genetic changes in the genes expressed in adults (either in the same gene or
518 in equivalent gene functions) matching the phenotypic convergence observed in dietary
519 specialist *Dysdera*. Nevertheless, it is largely known that hemiplasy can also produce such
520 matching patterns, inducing false evidence of convergent evolution (Mendes et al., 2016; Wu
521 et al., 2018). Indeed, the high level of gene tree discordance caused by ancestral
522 polymorphisms could potentially explain some of the repeated changes identified in *D.*
523 *gomerensis* and *D. tilosensis*. Nonetheless, some lines of evidence support that most of the
524 candidates reported in this study accumulated convergent changes in specialist lineages. First,
525 for realistic effective population sizes (i.e., $10^4 < N_e \leq 10^5$; these spiders are island endemic
526 predators with likely low census sizes), the probability of observing discordant trees
527 matching the phenotypic convergence is very low (Figure 3). The estimates of the HRF
528 values in branch B under realistic effective population sizes ranged from 0.001 to 0.134
529 (Figure 3b and 3c). Therefore, the probability of occurrence of ILS on this branch,
530 accompanied by a mutation in the branch A or in an older lineage creating a false pattern of
531 homoplasy, is much lower than that of true homoplasy (Guerrero &, Hahn, 2018). Second,
532 among the total set of discordant gene trees with high bootstrap support, only the 1.69% (62
533 out of 3,666) yielded resolved topologies that match exactly the one expected from
534 convergence in specialists, which agrees with hemiplasy risk predictions for intermediate
535 effective population sizes. Even so, and to be conservative, we excluded from the

536 downstream functional prediction analysis all candidates with gene trees included in this
537 1.69%. This approach, however, may not be suitable for detecting convergent changes in
538 gene expression in specialists. Actually, the assumption that the regulatory regions
539 responsible of the concordant changes in gene expression of candidate genes are completely
540 linked to the transcribed sequence (i.e., both share the same gene tree) may not be correct.
541 Estimates of the recombination rate in these genomes are not available and, more
542 importantly, some of these mutations could be far away from the coding region, even acting
543 in *trans*. In these cases, however, we would expect that gene-tree discordance will be
544 randomly distributed across the genome. We found, by contrast, a clear bias in our candidates
545 towards genes and functions biologically relevant for dietary specialists. Bearing all this in
546 mind, the fixation of convergent genetic changes remains as the most likely explanation for
547 most of the discordant patterns matching phenotypic convergence, even for MGE candidates.
548 Consequently, we demonstrated that our study design, with two evolutionary replicates of the
549 same dietary specialization event, was able to identify potential candidate genes and groups
550 of functionally equivalent genes responsible in part to these remarkable ecological shifts.
551
552 A priori, we would expect that the biological functions targeted by selection are related to
553 prey capture and food assimilation, both in digestive and metabolic aspects. Since genetic
554 changes underlying morphological modifications of the specialists' mouthparts likely involve
555 changes in gene expression patterns during development, they were undetectable in our
556 comparative analysis of adult transcriptomes. However, other aspects related to the detection,
557 attack, consumption and digestion of a prey with remarkable behavioural and chemical
558 defences definitely played a crucial role in specialization. Several studies have revealed
559 significant differences in the growth and nutrient extraction efficiencies in specialist *Dysdera*
560 fed on woodlouse, which suggests the existence of metabolic adaptations (Řezáč & Pekár,

2007; Toft & Macías-Hernández, 2017; Macías-Hernández et al., *in prep*.). Toxicity is the most relevant nutritional aspect that makes isopods a prey commonly rejected by most generalist spiders (Hopkin & Martin, 1985). Indeed, isopods accumulate toxic substances, including high concentrations of heavy metals from the soil, especially copper but also zinc, lead and cadmium, in vesicles such as lysosomes (Paoletti & Hassall, 1999). The toxic effects as well as some of the underlying genetic response mechanisms of heavy metals on terrestrial invertebrates have been known for a long time (Janssens, Roelofs, & van Straalen, 2009; Merritt & Bewick, 2017; Migula, Wilczek, & Babczyńska, 2013). Remarkably, our results are in full agreement with the few comparative transcriptomics studies conducted on these types of animals under different metal-stress conditions (e.g. Gomes, Scott-Fordsmand, & Amorim, 2014; Roelofs et al., 2009; Zapata, Tanguy, David, Moraga, & Riquelme, 2009), including in spiders (Li et al., 2016). These studies demonstrate that arthropods exposed to heavy metals show important gene expression changes relative to controls; remarkably, some of the reported gene targets also appear among our MGE candidates or correspond with some of the molecular functions enriched in our list. Some examples include ABC transporters, amiloride-sensitive sodium channels, ATPases, MAP kinases, ubiquitin ligases, histones, members of the cytochrome P450 family and ribosomal proteins (Table S4). These consistent results across different studies on phylogenetically distant species, support the idea of a relatively well-conserved common mechanism for the tolerance of heavy metal toxicity across animals. The old origin of such an evolutionary mechanism validates our approach for identifying the genetic determinants of stenophagy in *Dysdera.*

Genetic changes matching phenotypic convergence: metal-induced damage or adaptive response to metal stress?

585 We found that most MGE candidates were specifically downregulated in specialists and
586 encoded molecular functions involved in cell response, vesicular transport, organization of
587 organelles and cytoskeleton, cilia assembly, or cell adhesion (Table S4). Noticeably, these are
588 the most frequent cell modifications observed in intestinal tissue damage by heavy metals
589 from the diet (e.g., Bednarska et al., 2016; Köhler & Alberti, 1992; Zhang et al., 2001).
590 Indeed, in soil arthropods subjected to heavy-metal stress, midgut cells show evident
591 histological modifications indicative of metal deposition in intracellular granules and gut
592 epithelial degeneration. Although the downregulation pattern observed in specialist *Dysdera*
593 could be the result of a direct stress-induced perturbation of gene expression caused by the
594 high concentration of heavy metals supplied in a woodlouse-rich diet, they might actually be
595 part of an adaptive biological response to excrete metals or other toxic substances more
596 efficiently, thus avoiding their assimilation (Van Straalen & Roelofs, 2005). Consistent with
597 this hypothesis, we observed concordant DE patterns in some MAP kinase pathway members,
598 which participate in an important stress-activated/immune response cascade (Chmielowska-
599 Bąk & Deckert, 2012), and in some ubiquitin ligases, which, among other functions, are
600 involved in the inhibition of cell growth and cycle arrest in response to DNA damage (Cao &
601 Yan, 2012). The adaptive response in specialists would consist of downregulating a set of
602 genes to keep gut epithelial cells in a semi-degenerated functional and structural state that
603 allows enhanced accumulation of heavy metals in granules and very fast and effective
604 intestinal exfoliation and regeneration.
605
606 Our analysis also uncovered a number of upregulated MGE and MPS candidates associated
607 with iron, copper and zinc binding and homeostasis, which can also be part of an adaptive
608 mechanism of detoxification in specialist *Dysdera.* Among these candidates, we found
609 amiloride-sensitive sodium channels, membrane ATPases and ABC and dicarboxylate

transporters. These proteins are either antiporters for metal cations or are involved in cellular mechanisms for heavy metal vacuolar sequestration (Ahearn, Sterling, Mandal, & Roggenbeck, 2010) or in cellular metal homeostasis and detoxification (e.g., Sooksa-Nguan et al., 2009; Lee et al., 2014). Another set of interesting candidates are the proteins annotated as syntaxin-5-like proteins with a SNARE domain, which are involved in vesicle tethering and fusion associated with copper ion homeostasis (Norgate et al., 2010) and, in addition to being significantly overexpressed in both specialists, also show signals of positive selection in *D. tilosensis*.

It is well known that heavy metal-associated toxicity is largely due to damage to the oxidative tissue caused by the accumulation of reactive oxygen species in the cell (Schieber & Chandel, 2014). Noticeably, among the upregulated MGE candidates (and those regulated in only one of the specialists), we found members of family 3 of the P450 cytochromes, a group of monooxygenases that constitute the largest and most functionally diverse class of insect detoxification enzymes and that have been implicated in the oxidative detoxification of furanocoumarins, alkaloids, plant secondary metabolites and synthetic insecticides (Nelson & Nebert, 2011). Additionally, we identified among the candidates several esterases, a group of proteins with a role in heavy metal and pesticide detoxification that have been used as biomarkers of metal exposure in many organisms, including spiders (Wilczek, Babczyńska, Migula, & Wencelis, 2003). We identified esterases significantly overexpressed in both specialists, although in this case, the orthogroups of *D. gomerensis* and *D. tilosensis* were different, suggesting possible convergence at the functional level rather than at the gene level. Remarkably, two of these esterases also showed a positive selection signal in *D. gomerensis*.

We also detected other MGE candidates associated with the metabolism of some essential nutrients, such as proteins with chitin-binding and chitinase activity, and enzymes involved in the metabolism of amino acids, sugars and lipids. Given that most of these candidates were downregulated in specialists, the adaptive advantage could be associated with a reduction in biosynthetic processes to save energy, presumably to dedicate the energy to detoxification processes. However, the presence of some upregulated and positively selected genes among these metabolic candidates indicates that specialists might also have developed an adaptive mechanism to enhance the assimilation and metabolization of some other nutrients present in woodlice but less accessible to other preys.

Finally, it is worth noting that MPS candidates are also significantly enriched in genes related with the immune system. It has been reported that high concentrations of heavy metals negatively affect important processes, such as phagocytosis and chemotaxis, during the generation of the immune response (Boyd, 2010). The footprint of positive selection detected in specialist *Dysdera*, matching phenotypic divergence, might reflect an adaptive mechanism to alleviate the negative immunomodulation effects of heavy metals. In fact, there is evidence that positive selection promoted local adaptation of herbivore insects to heavy metal polluted environments by enhancing immune functions (van Ooik & Rantala, 2010) suggesting the important adaptive character of this system under metal-stress conditions.

A possible role of venom toxins in the convergent dietary shift

Stenophagous spiders (e.g., myrmecophagous, termitophagous and araneophagous spiders) show increased venom toxicity to the preferred prey, while related generalists show similar toxicities to all preys (Pekár, Líznarová, Bočánek, & Zdráhal, 2018). The analysis of venom components in stenophagous species indicates that this difference in efficacy is caused by the

presence of prey-specific toxins, suggesting evolutionary adaptations for more effective exploitation of focal prey. Notably, we identified a number of transcripts encoding venom toxins among the MGE candidates, most of which were upregulated in specialists, an opposite pattern to that obtained for the rest of the MGE candidates. Among others, we found candidates encoding astacin-like metalloproteases. Astacins share common features with serralysins, matrix metallo-endopeptidases, and snake venom proteases and might be involved in the proteolytic processing of other venom toxins or even play a role in extra-oral digestion of prey, which could be important in the specialization of Canarian *Dysdera* to woodlice. Interestingly, the MGE candidates encoding astacin-like metalloproteases belonged to different orthogroups in each specialist species, which suggests an additional example of functional convergence through different genes. Our analysis also uncovered other candidates that encode some lesser-known toxins, such as products with sequence similarity to U24-ctenitoxin-Pn1a (presumably a protease inhibitor), pisautoxin-Dm1a (a toxin from the venom of the spider *Dolomedes mizhoanus* with an unknown target), alpha-latrotoxins (which induce massive neurotransmitter release) and aranetoxins (also with an unknown target). Remarkably, we found that among the alpha-latrotoxins, a transcript with similarity to a crustacean-selective component of spider venom (the alpha-latrocrustatoxin; Grishin, 1998), also showed the signature of positive selection, making it a promising candidate for stenophagy. Further research including venom gland-specific transcriptomes and the study of venom toxicity to different preys would be required to shed light on the role of venom in the convergent dietary specialization of *Dysdera*.

Repeated adaptation to stenophagy in Canarian endemic *Dysdera*: collateral or parallel evolution?

683 Here, we uncovered several pieces of evidence supporting the adaptive divergence hypothesis
684 in stenophagous *Dysdera* inhabiting Western Canary Islands. First, the functional annotation
685 of the majority of genes with concordant changes in gene expression between generalist and
686 specialist spiders clearly points towards an active role of these genes in the dietary shift.
687 Second, we detected repeated episodes of positive selection in the same genes (or
688 functionally related group of genes) in the two specialists' lineages. Furthermore, a
689 significant number of MPS candidates showed convergent amino acid preference shifts in the
690 two focal branches, some of which were also inferred to be under positive selection.
691 Altogether, these results provide new significant evidence that species can find the same
692 molecular solutions to adapt predictably to similar ecological niches more often than
693 previously thought (see Marques et al., 2017; Nosil et al., 2018, for other recent examples).
694
695 Specialist *Dysdera* may have repeatedly adapted to stenophagy through parallel or collateral
696 evolution. In the first case, convergence would result from the accumulation of the same or
697 similar mutations in evolutionary independent lineages, whereas in the second, selection on
698 either shared ancestral or introgressed variations, would be the responsible of the convergent
699 patterns (Stern, 2013). In recent years, increasing evidence has emerged suggesting the
700 important role of shared genetic variation as a substrate for driving repeated evolution of
701 ecotypes in nature (e.g. Jones et al., 2012; Marques, Meier, & Seehausen, 2019; Schluter &
702 Conte, 2009; Van Belleghem et al., 2018). Our genome-wide HRF and D_{FOIL} analyses point
703 to that most of our candidates originated from parallel independent evolution (i.e., relatively
704 low risk of random ILS and non-significant D_{FOIL} results). On the other hand, in the five
705 positive selection candidates where the individual gene trees were incongruent, the apparent
706 homoplasy could be the result of collateral evolution. Unfortunately, in these cases, current
707 data would not allow to disentangle collateral evolution from random ILS at the individual

gene level. Accordingly, and to avoid reporting candidates with false patterns of homoplasy, we excluded these five genes with discordant topologies, restricting the analysis on the parallel fixation of *de novo* mutations. Further research including polymorphism from whole genome data would be needed to unequivocally establish the relative role of collateral evolution in the convergence observed in these island endemic spiders.

Altogether, our findings suggest that the ecological opportunity provided by the colonization of the Canary Islands facilitated the exploration of multiple adaptive landscapes by *Dysdera* and its diversification on similar peaks (Mahler, Ingram, Revell, & Losos, 2013), providing an exceptional example of repeatability in evolution and shedding light on the genetic determinants of phenotypic convergence (Stroud & Losos, 2016). Besides, our results support the idea that convergence can involve repeated changes at different hierarchical levels (Rosenblum, Parent, & Brandt, 2014). We found convergent changes at the amino acid, gene and gene function levels that would be mostly associated to the excretion and detoxification of heavy metals accumulated in the preferred prey, and some venom components likely related with prey capture. We also demonstrated that natural selection promoted the fixation of some of these changes, confirming the view that adaptive forces are a primary determinant of phenotypic convergence (Storz, 2016). Moreover, our report uncovering repeated genetic changes in pairs of phylogenetically-close taxa, supports the ongoing debate that the probability of shared molecular changes for convergent phenotypes correlates with node age (Conte, Arnegard, Peichel, & Schluter, 2012). Hence, this study not only provides new evidence on the genomic basis of an extraordinary example of a convergent ecological shift in a non-model organism but also offers new insights into the longstanding debate about predictability in evolution.

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Authors' contributions

A.S-G. and J.R. designed, conceived and supervised the research; N.M-H and M.A.A. provided the biological material. J.V. performed the experiments and the bioinformatics work, and analysed the data. M.A.A. performed the dissecting analysis and participated in the data interpretation. J.V., J.R. and A.S-G. wrote the first version of the manuscript. N.M-H. and M.A.A. revised the manuscript and participated in the writing of the final version. All authors read and approved the final version of the manuscript.

Data accessibility

The raw sequence data generated for this work has been deposited at the Sequence Read Archive (SRA) under Bioproject PRJNA437566. Additional data and analysis generated in this study have been deposited in Figshare (https://doi.org/10.6084/m9.figshare.7726508.v1).

Competing interests

The authors declare that they have no competing interests

Figures

Figure 1. a. Map of the Canary Islands showing the geographic location of capture localities. **b.** Phylogenetic relationships and divergence times (scale bar) among surveyed *Dysdera* species. The continental species *D. crocata* was used to root the tree. **c.** Dissecting scope images of the left chelicera: A-B: *Dysdera silvatica* female, La Gomera, A, ventral view; B, lateral view; C-D: *D. verneaui* female, Tenerife, C, ventral view, D, lateral view; E-F: *D. bandamae* female, Gran Canaria, E, ventral view, F, lateral view; G-H: *D. gomerensis* female, La Gomera, G, ventral view, H, lateral view; I-J: *D. tilosensis* male, Gran Canaria, I, lateral view, J, lateral view. Bars indicate the relative lengths of the different parts of the chelicerae to highlight differences between the standard (generalists) and elongated or slightly elongated (specialists) chelicerae. White bar: total length of the basal segment (b), dotted part: length of the cheliceral groove (g). Black bar: length of the cheliceral fang (f). In standard chelicerae, g is approximately 1/3 of b, and f is similar to the distance between the base of the segment and the end of the internal keel (k), while in elongated chelicerae, g is longer than 2/5 of f, and f is longer than k. Scale bar in mm. **d.** Live images of the target *Dysdera* species; photo credit: Pedro Oromí.

Figure 2. Core analyses workflow applied in this study, including a summary of the most relevant results. DE, differential expression; DFC, differential functional constraints; PS, positive selection; *, patterns matching the observed phenotypic convergence.

Figure 3. Species tree inferred with Astral showing the risk of hemiplasy along the phylogeny. Hemiplasy risk factor values (HRF) were estimated for all internal branches of the tree. The relative probabilities of hemiplasy and homoplasy were inferred under different

effective population sizes (N_e; panels **a** to **d**) and assuming a fixed mutation rate μ per $2N_e$ generations ($2N_e\mu = 5.5 \times 10^{-3}$). HRF values estimated for all internal branches (in brackets) represent the proportion of discordant traits associated with a branch due to hemiplasy.

Figure 4. Heat map with body part-specific gene expression profiles of the 144 MGE candidates.

Figure 5. Bar charts with the most relevant results of the GO enrichment analyses (see Figure S3 for more detailed versions). a**.** Orthogroups with differential expression profiles matching phenotypic convergence (144 MGE candidates) **b.** Orthogroups under positive selection in the two specialists (17 MPS candidates) **c.** Most representative candidates encoding venom toxins in stenophagous *Dysdera*. Dark and light tones represent the proportion of genes with a given associated GO in the candidate and the population (whole transcriptome) set, respectively.

Figure 6. Relevant orthogroups showing evidence of convergent amino acid substitutions. (**a**) orthogroup encoding the venom toxin OG6752. (**b-f**) orthogroups with positions evolving under positive selection. Amino acid positions are shaded with different tones according to their profiles, and only positions with a *PP* equal to or greater than 0.99 according to the PCOC, PC or OC model are shown (Rey et al., 2018). Stars highlight the sites identified as being positively selected in MEME.

Tables

Table 1. Summary of dietary habits, sampling localities, RNA-seq data and assembly statistics for each surveyed *Dysdera* species.

Supplementary material

Supplementary figures

Figure S1. Distribution of blastx hits across species. Distribution of the top 5 hits from the blastx searches with the transcripts of each *Dysdera* species against the ArthropodDB database.

Figure S2. Principal component analysis (PCA) of gene expression profiles of individual *REST* samples from *D. tilosensis*.

Figure S3. Venn diagrams showing (**a**) the number of shared genes between species pairs. Differential expressed (DE) genes are showed in brackets; (**b**) the number of DE genes between species pairs and groups of tissues (*LEGS-PALP* refers to the *LEG#1*, *LEG#234* and *PALP*); (**c**) number of MGE candidates across tissues.

Figure S4. Tree maps with detailed GO enrichment results generated with REVIGO.

Figure S5. Box plots showing the distribution of ω values for all single-copy orthogroups in specialist (orange) and generalist (blue) species.

Figure S6. Orthogroups with evidence of convergent amino acid evolution. Amino acid positions are coloured according to their profiles, and only positions with a *PP* equal to or greater than 0.99 according to the PCOC, PC or OC model are shown. Yellow stars highlight the sites identified as positively selected in MEME.

Supplementary tables

Table S1. RNA-seq statistics.

Table S2. Distribution of the percentage of CEG length covered by blastx hits.

Table S3. Orthogroups classification.

Table S4. List of genes with concordant differential expression profiles between generalist and specialists species.

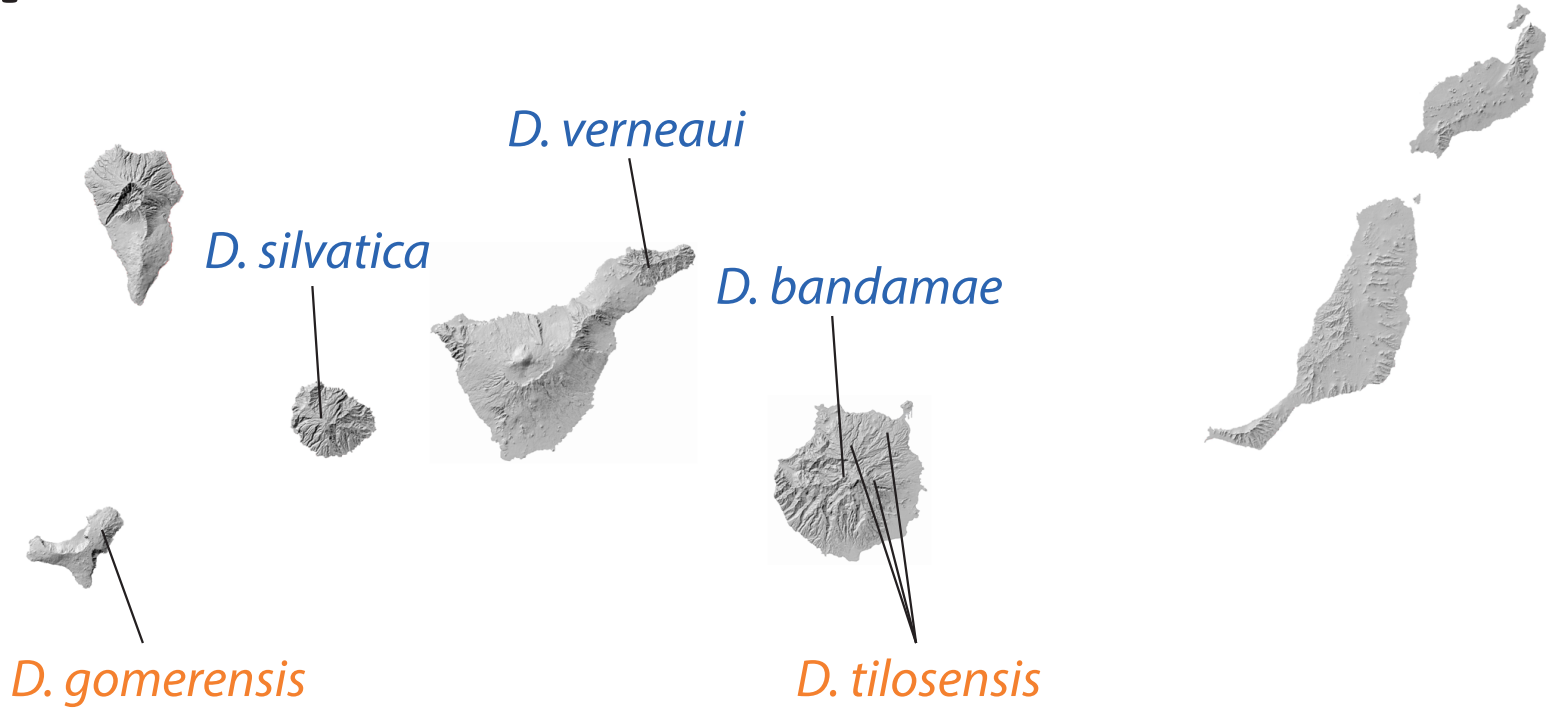
Table S5. List of genes with concordant differential functional constraint profiles between generalist and specialist species.

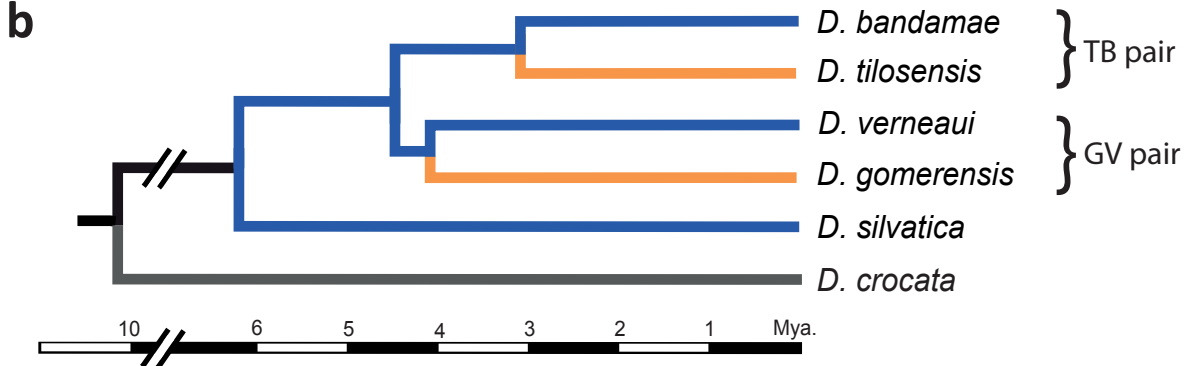
Table S6. List of genes with concordant signals of positive selection in specialist species.

Supplementary methods

Supplementary Information of transcriptome, differential gene expression and selective constraint analyses.

a


D. verneaui
D. silvatica
D. bandamae
D. gomerensis
D. tilosensis


b
D. bandamae
D. tilosensis
TB pair
D. verneaui
D. gomerensis
GV pair
D. silvatica
D. crocata
10
6
5
4
3
2
1
Mya.

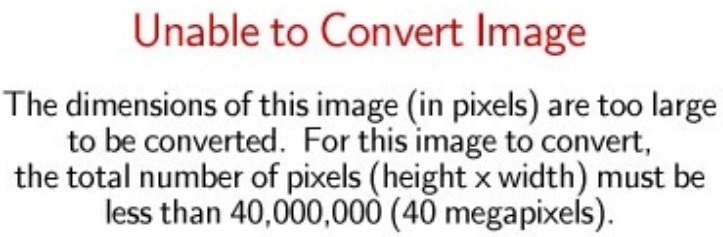
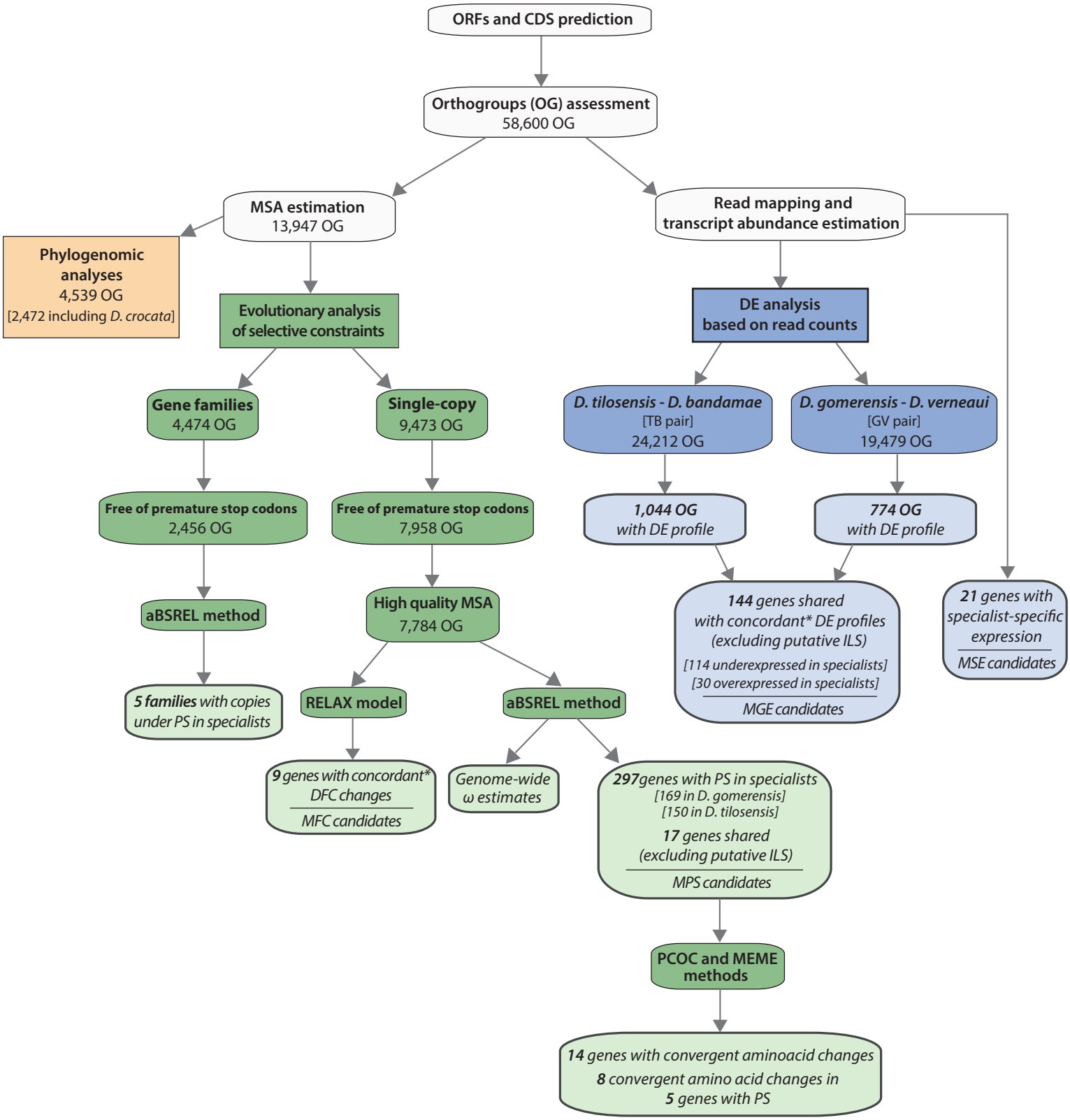
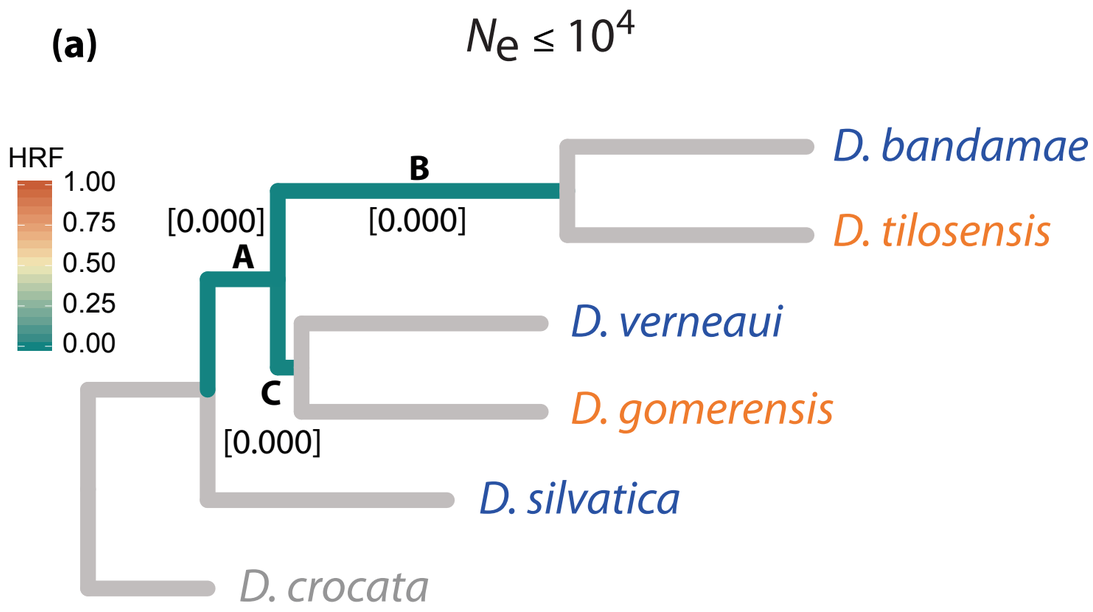
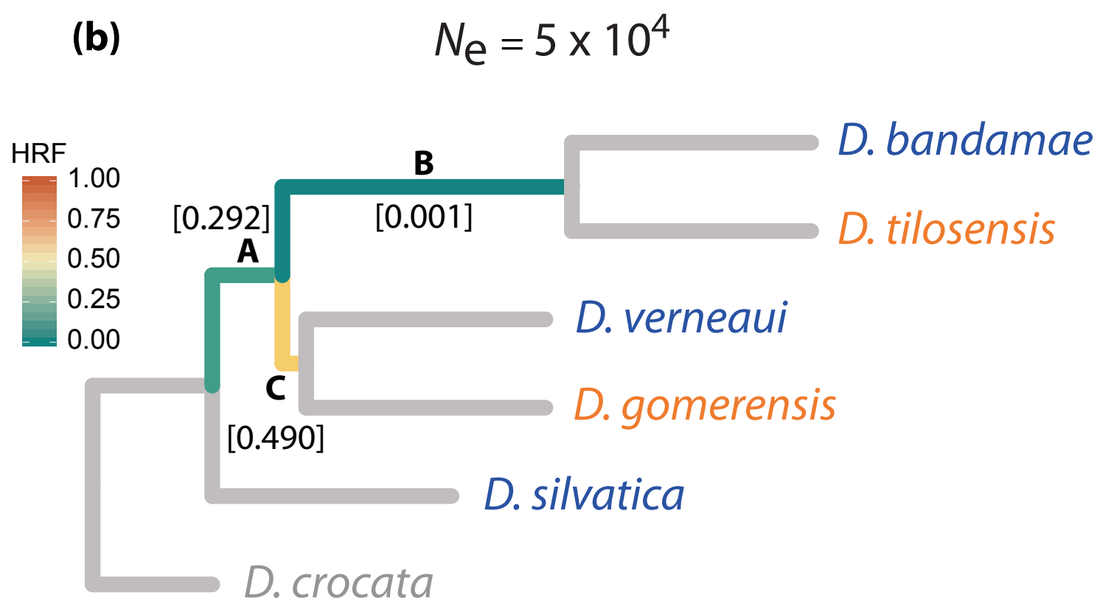
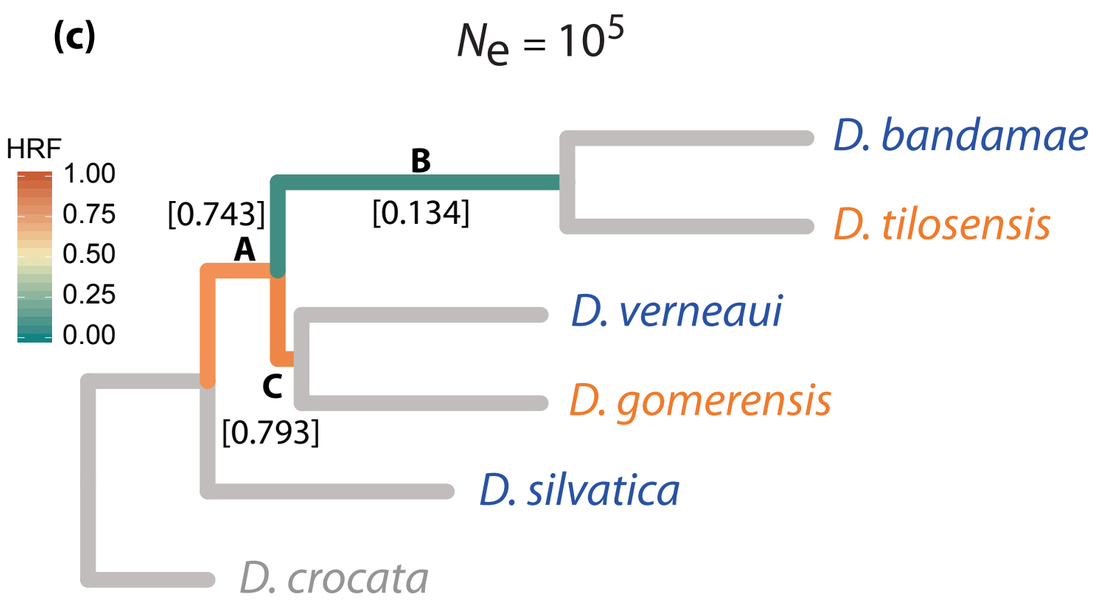

Unable to Convert Image
The dimensions of this image (in pixels) are too large
to be converted. For this image to convert,
the total number of pixels (height x width) must be
less than 40,000,000 (40 megapixels).

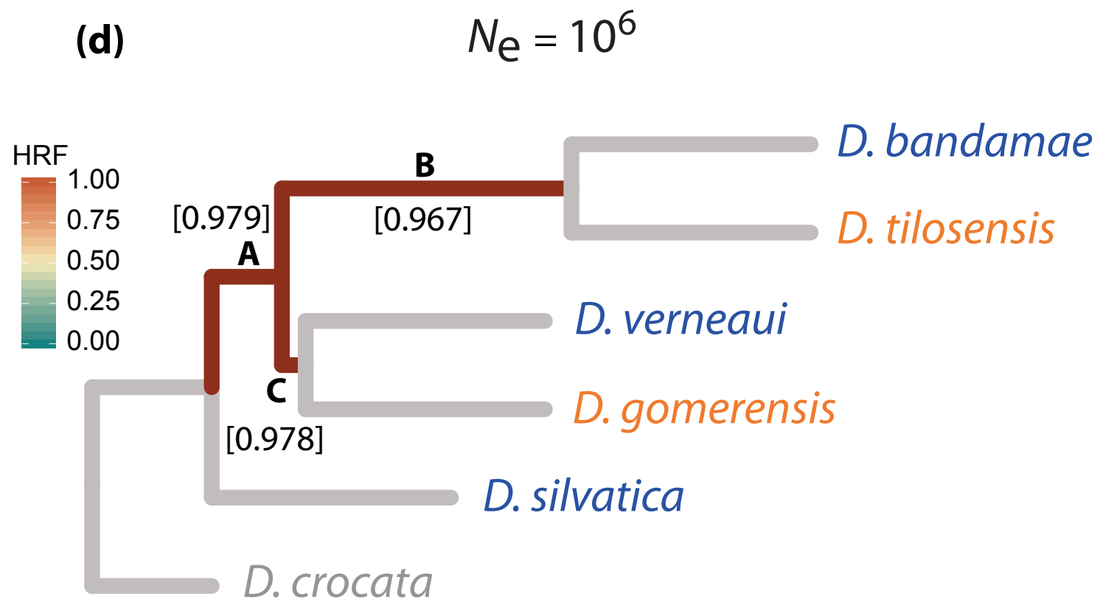
Figure 1cd

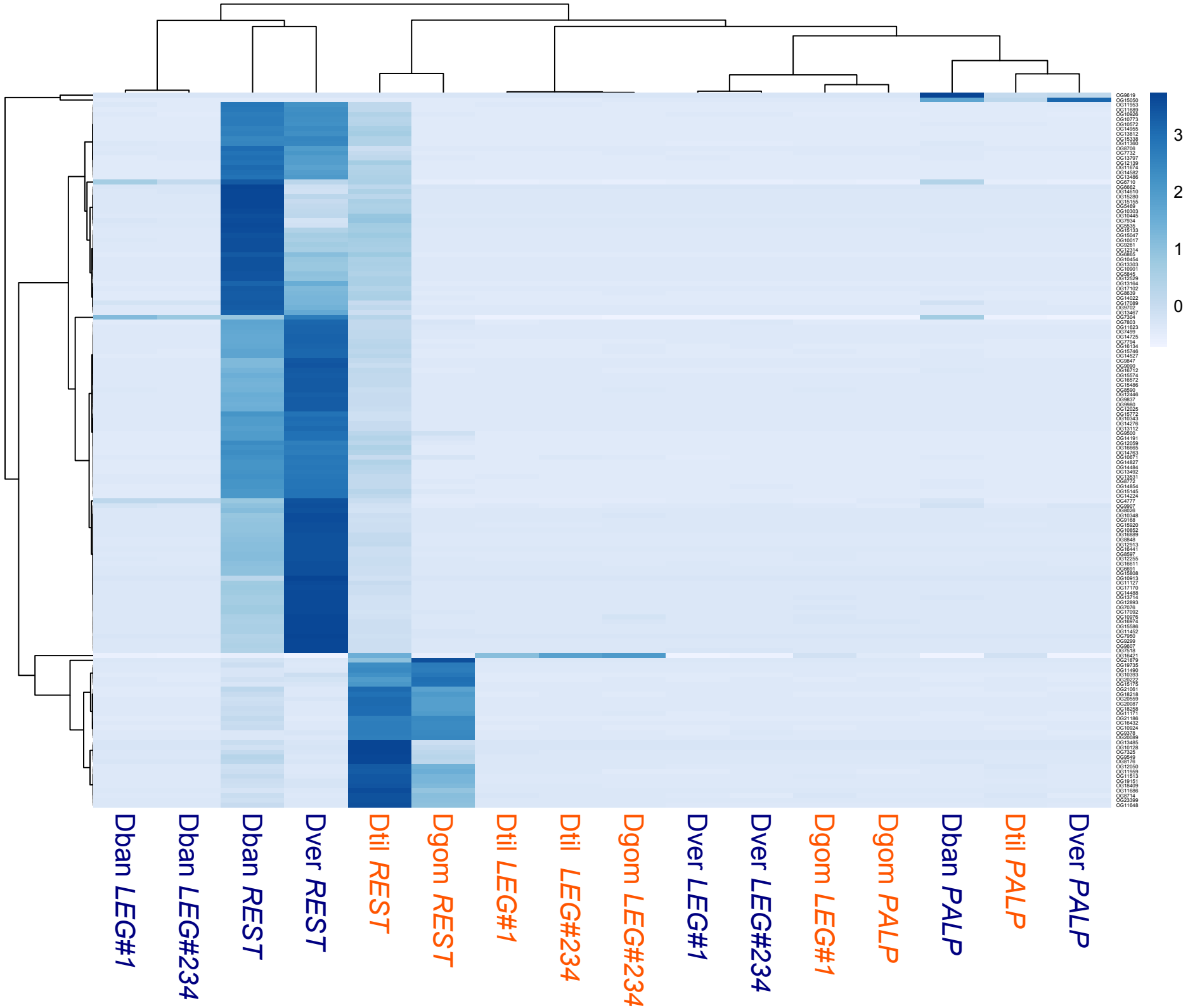

ORFs and CDS prediction
Orthogroups (OG) assessment
58,600 OG
MSA estimation
13,947 OG
Read mapping and transcript abundance estimation
Phylogenomic analyses
4,539 OG
[2,472 including D. crocata]
Evolutionary analysis of selective constraints
DE analysis based on read counts
Gene families
4,474 OG
Single-copy
9,473 OG
D. tilosensis - D. bandamae
[TB pair]
24,212 OG
D. gomerensis - D. verneaui
[GV pair]
19,479 OG
Free of premature stop codons
2,456 OG
Free of premature stop codons
7,958 OG
1,044 OG
with DE profile
774 OG
with DE profile
aBSREL method
High quality MSA
7,784 OG
144 genes shared
with concordant* DE profiles
(excluding putative ILS)
[114 underexpressed in specialists]
[30 overexpressed in specialists]
MGE candidates
21 genes with specialist-specific expression
MSE candidates
5 families with copies under PS in specialists
RELAX model
aBSREL method
9 genes with concordant* DFC changes
MFC candidates
Genome-wide ω estimates
297 genes with PS in specialists
[169 in D. gomerensis]
[150 in D. tilosensis]
17 genes shared
(excluding putative ILS)
MPS candidates
PCOC and MEME methods
14 genes with convergent aminoacid changes
8 convergent amino acid changes in
5 genes with PS

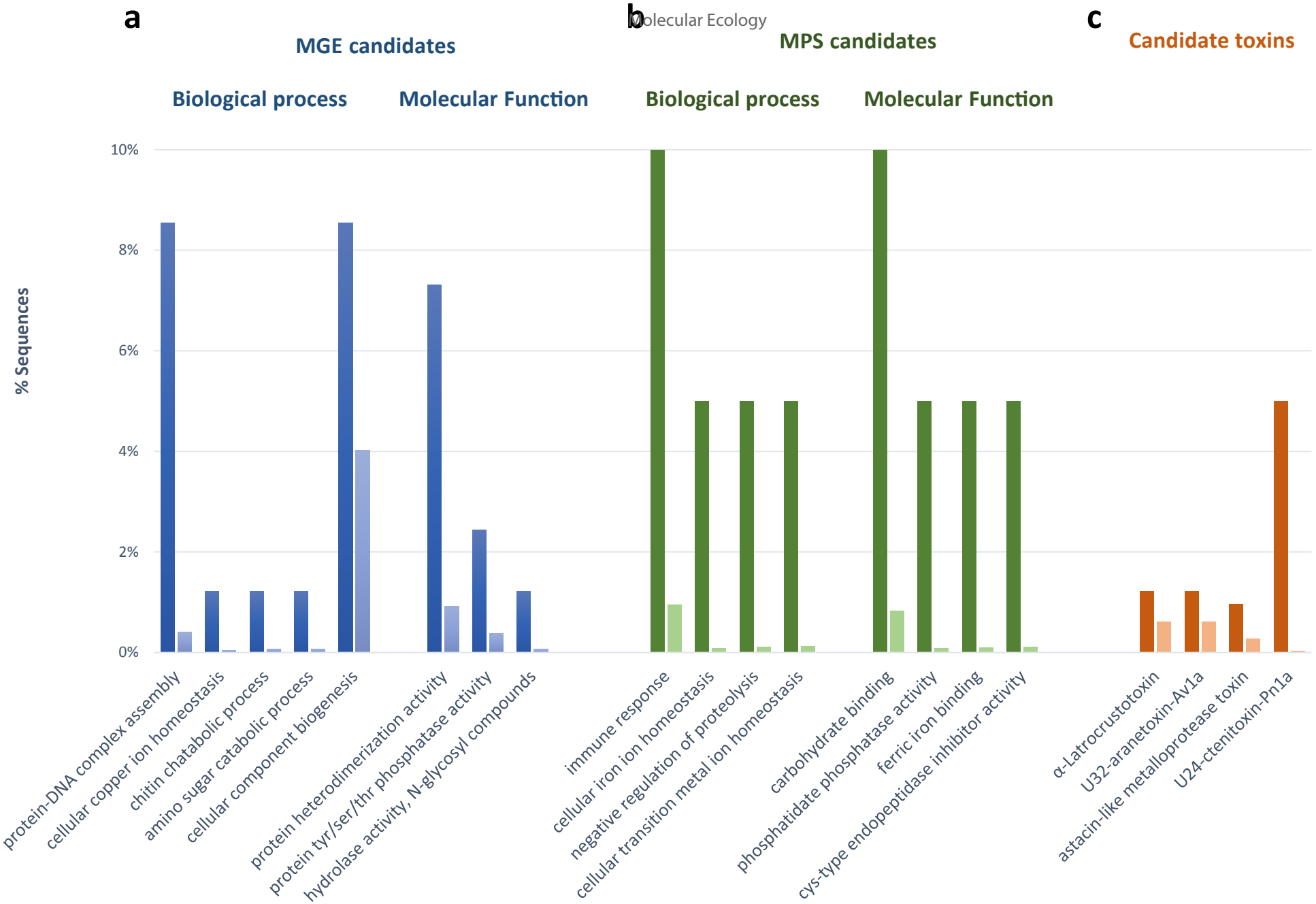

(a)
$N_e \leq 10^4$
HRF
1.00
0.75
0.50
0.25
0.00
[0.000]
A
B
[0.000]
C
[0.000]
D. bandamae
D. tilosensis
D. verneaui
D. gomerensis
D. silvatica
D. crocata


(b)
$N_e = 5 \times 10^4$
HRF
1.00
0.75
0.50
0.25
0.00
[0.292]
A
B
[0.001]
C
[0.490]
D. bandamae
D. tilosensis
D. verneaui
D. gomerensis
D. silvatica
D. crocata


(c)
$N_e = 10^5$
HRF
1.00
0.75
0.50
0.25
0.00
[0.743]
A
B
[0.134]
C
[0.793]
D. bandamae
D. tilosensis
D. verneaui
D. gomerensis
D. silvatica
D. crocata


(d)
$N_e = 10^6$
HRF
1.00
0.75
0.50
0.25
0.00
[0.979]
A
B
[0.967]
C
[0.978]
D. bandamae
D. tilosensis
D. verneaui
D. gomerensis
D. silvatica
D. crocata


3
2
1
0
Dban LEG#1
Dban LEG#234
Dban REST
Dver REST
Dtil REST
Dgom REST
Dtil LEG#1
Dtil LEG#234
Dgom LEG#234
Dver LEG#1
Dver LEG#234
Dgom LEG#1
Dgom PALP
Dban PALP
Dtil PALP
Dver PALP


a
MGE candidates
Biological process
Molecular Function
b
MPS candidates
Biological process
Molecular Function
c
Candidate toxins
% Sequences
10%
8%
6%
4%
2%
0%
protein-DNA complex assembly
cellular copper ion homeostasis
chitin chatabolic process
amino sugar catabolic process
cellular component biogenesis
protein heterodimerization activity
protein tyr/ser/thr phosphatase activity
hydrolase activity, N-glycosyl compounds
immune response
cellular iron ion homeostasis
negative regulation of proteolysis
cellular transition metal ion homeostasis
carbohydrate binding
phosphatidate phosphatase activity
ferric iron binding
cys-type endopeptidase inhibitor activity
α-Latrocrustotoxin
U32-aranetoxin-Av1a
astacin-like metalloprotease toxin
U24-ctenitoxin-Pn1a

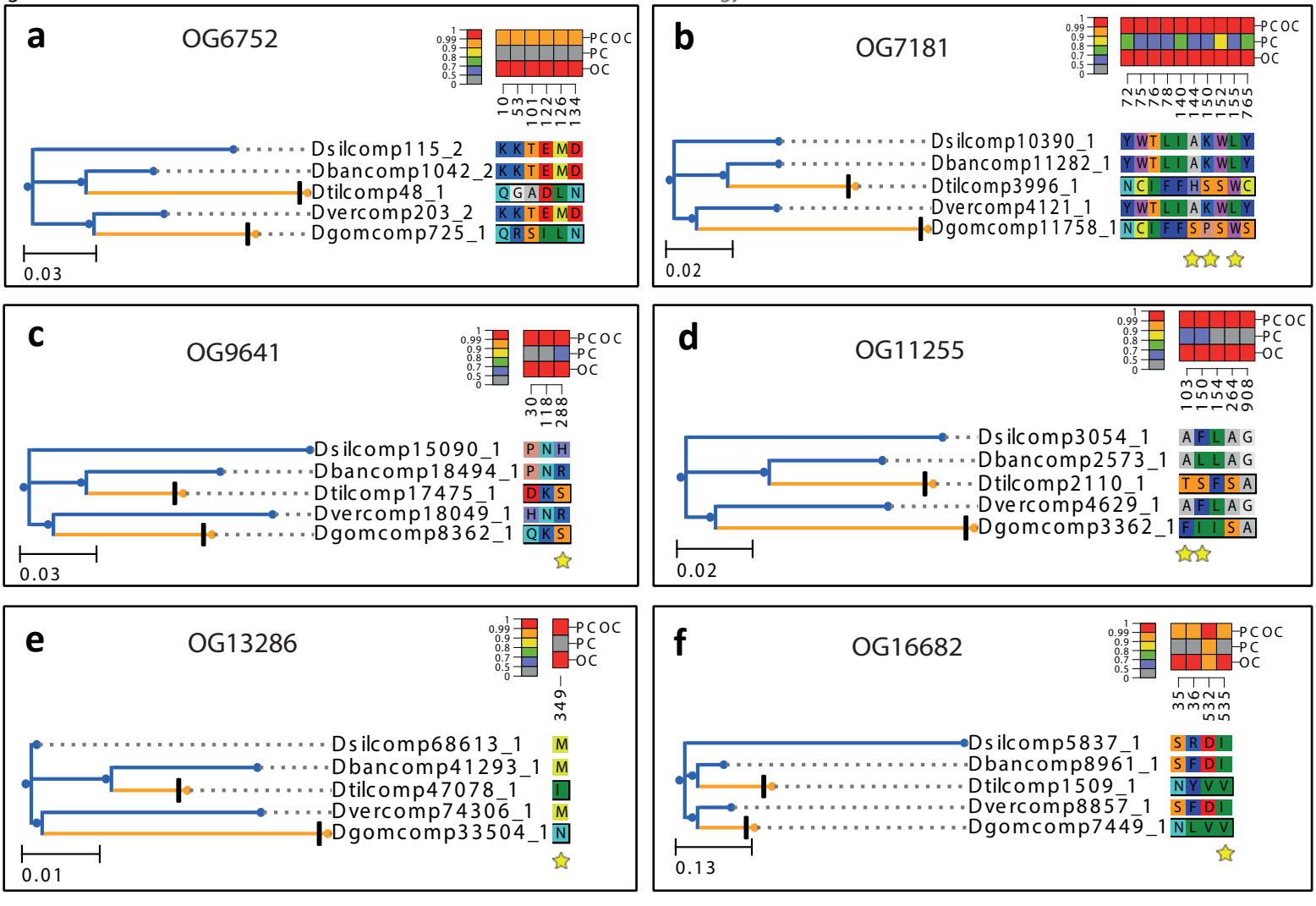

a
OG6752
PCOC
PC
OC
10 53 101 122 126 134
Dsilcomp115_2 K K T E M D
Dbancomp1042_2 K K T E M D
Dtilcomp48_1 Q G A D L N
Dvercomp203_2 K K T E M D
Dgomcomp725_1 Q R S I L N
0.03
b
OG7181
72 75 76 78 140 144 150 152 155 765
Dsilcomp10390_1 Y W T L I A K W L Y
Dbancomp11282_1 Y W T L I A K W L Y
Dtilcomp3996_1 N C I F F H S S W C
Dvercomp4121_1 Y W T L I A K W L Y
Dgomcomp11758_1 N C I F F S P S W S
0.02
c
OG9641
30 118 288
Dsilcomp15090_1 P N H
Dbancomp18494_1 P N R
Dtilcomp17475_1 D K S
Dvercomp18049_1 H N R
Dgomcomp8362_1 Q K S
0.03
d
OG11255
103 150 154 264 908
Dsilcomp3054_1 A F L A G
Dbancomp2573_1 A L L A G
Dtilcomp2110_1 T S F S A
Dvercomp4629_1 A F L A G
Dgomcomp3362_1 F I I S A
0.02
e
OG13286
349
Dsilcomp68613_1 M
Dbancomp41293_1 M
Dtilcomp47078_1 I
Dvercomp74306_1 M
Dgomcomp33504_1 N
0.01
f
OG16682
35 36 532 535
Dsilcomp5837_1 S R D I
Dbancomp8961_1 S F D I
Dtilcomp1509_1 N Y V V
Dvercomp8857_1 S F D I
Dgomcomp7449_1 N L V V
0.13

Table 1. Summary of dietary habits, sampling localities, RNA-seq data and assembly statistics for each surveyed *Dysdera* species

	D. silvatica	*D. verneaui*	*D. gomerensis*	*D. bandamae*	*D. tilosensis*
Diet	Generalist	Generalist	Specialist	Generalist	Specialist
Locality (in Canary Island)	La Gomera	Tenerife	El Hierro	Gran Canaria	Gran Canaria
Total raw reads	441,835,864	527,299,202	430,522,240	765,653,462	678,150,384
Total qualified reads	418,205,054	495,937,054	400,095,710	746,925,920	664,654,842
Transcripts	236,283	441,604	213,984	296,544	316,498
Genes (clustered isoforms)	170,846	347,878	177,363	221,801	229,762
Gene average length (in bp)	702	525	622	658	649
Gene maximum length (in bp)	26,709	27,235	27,386	27,369	25,342
HK genes	1,136	1,194	1,232	1,153	1,159
CEG genes	807 (457)	1,180 (457)	1,111 (457)	1,033 (457)	1,143 (457)
GO annotated genes	29,879	38,361	28,158	35,116	37,246
Genes with InterPro domain	30,886	40,771	29,930	37,413	39,480
Functional annotated genes[a]	31,091	41,019	30,106	37,620	39,704
Annotated genes[b]	41,046	51,864	37,087	47,059	50,150
Predicted coding sequences (CDS)	58,966	84,114	55,914	72,352	77,756
% not coding genes	34.51%	24.18%	31.53%	32.62%	33.84%
% not annotated CDS	69.61%	61.66%	66.33%	65.04%	64.50%
1to1 orthologs in all species	9,473	9,473	9,473	9,473	9,473
1to1 orthologs per species pair	-	19,497	19,497	24,212	24,212

[a] GO or Interpro hits.

[b] GO, Interpro or blast hits.