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**A genome scan of diversifying selection in *Ophiocordyceps* zombie-ant fungi
suggests a role for enterotoxins in coevolution and host specificity**

Running title: **Diversifying selection in zombie-ant fungi**

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Abstract

Identification of the genes underlying adaptation sheds light on the biological functions targeted by natural selection. Searches for footprints of positive selection, in the form of rapid amino-acid substitutions, and the identification of species-specific genes have proved to be powerful approaches to identifying the genes involved in host specialization in plant-pathogenic fungi. We used an evolutionary comparative genomic approach to identify genes underlying host adaptation in the ant-infecting genus *Ophiocordyceps*, which manipulates ant behavior. A comparison of the predicted genes in the genomes of species from three species complexes – *O. unilateralis*, *O. australis* and *O. subramanianii* – revealed an enrichment in pathogenesis-associated functions, including heat-labile enterotoxins, among species-specific genes. Furthermore, these genes were overrepresented among those displaying significant footprints of positive selection. Other categories of genes suspected to be important for virulence and pathogenicity in entomopathogenic fungi (e.g., chitinases, lipases, proteases, core secondary metabolism genes) were much less represented, although a few candidate genes were found to evolve under positive selection. An analysis including orthologs from other entomopathogenic fungi in a broader context showed that positive selection on enterotoxins was specific to the ant-infecting genus *Ophiocordyceps*. Together with previous studies reporting the overexpression of an enterotoxin during behavioral manipulation in diseased ants, our findings suggest that heat-labile enterotoxins are important effectors in host adaptation and coevolution in the *Ophiocordyceps* entomopathogenic fungi.

Introduction

The identification of genes underlying adaptation is a major goal in evolutionary biology, as it can shed light on the biological functions targeted by natural selection and the genetic mechanisms generating new, adaptive variants. Innovation may be generated during evolution by gene duplication followed by rapid amino-acid substitutions in one of the copies (Ohno 1970, Zhang et al. 2002). Gene losses can also be adaptive (Juárez-Vázquez et al. 2017), particularly in pathogens, as the absence of a molecule recognized by the host may enable the pathogen to colonize its host without triggering a response from the host immune system (Albalat and Cañestro 2016; Ghanbarnia et al. 2015, Rouxel and Balesdent 2017). Gene duplications and losses result in the presence of species-specific genes, which are often over-represented among the genes involved in adaptation (Gladieux et al. 2014, Lespinet et al. 2002; Zhou et al. 2015). Adaptation may also occur through positive selection, with rapid amino-acid substitutions, typically detected as higher rates of non-synonymous substitutions (d_N) than of synonymous substitutions (d_S) among orthologous genes of closely related species (Kimura 1983; Ina 1996). Comparisons of d_N/d_S ratios to neutral expectations therefore also constitute a powerful approach to identifying genes under recurrent positive selection (Yang and Nielsen 1998; Yang et al. 2000, Yang et al. 2005).

Pathogens are particularly interesting models for investigations of the genomic mechanisms of adaptation, as they are locked in an arms race with their hosts, leading to continuous, rapid evolution (Anderson et al. 2010; Kurtz et al. 2016). Identification of the genes underlying host-specific adaptations in pathogens improves our fundamental understanding of natural selection and evolution, but it also has more applied implications, shedding light on major epidemics and disease emergence in plants and animals (Möller and Stukenbrock 2017).

Fungi are the principal pathogens of plants (Anderson et al. 2004), and they also represent threats to the health of many animals (Sexton and Howlett 2006; Fisher et al. 2012). Many studies have searched for genes under positive selection as a means of identifying genes and functions involved in the species-specific adaptation of fungal pathogens of plants (Aguileta et al. 2009; Möller and Stukenbrock 2017). For example, in the *Microbotryum* and *Botrytis* fungal plant pathogens, many such genes have been identified through comparative transcriptomics studies as being under recurrent positive selection and they were involved in biological processes important for the recognition and cell signaling between the host and the pathogen (Aguileta et al. 2010, 2012). More recently, next-generation sequencing made it possible to perform genome-wide scans in plant -pathogenic fungi, resulting in the identification of an array of effectors under positive selection (Badouin et al. 2017; Poppe et al. 2015; Schirmann et al. 2018; Stukenbrock et al. 2011; Wicker et al. 2013) and species- or lineages-specific genes underlying adaptations (Baroncelli et al. 2016, Hartmann et al. in press2018).

By contrast, far fewer such studies have been performed on entomopathogenic fungi (Wang and Wang 2017), despite the importance of identifying genes underlying host-specific adaptation for the use of these fungi as biological control agents against insect pests in agriculture (Wang and Feng 2014). Furthermore, an understanding of host-specificity and evolution in these insect pathogens is of fundamental interest in its own right, particularly for fungi able to manipulate the behavior of the insect host for their own benefit, as in the “zombie ant” phenomenon. Most genomic studies on entomopathogenic fungi have focused on species with agricultural applications such as *Beauveria bassiana* and *Metarhizium anisopliae* (Gao et al. 2011, Hu et al. 2014, Pattemore et al. 2014). However, these species have broad host ranges, and may not, therefore, be the best models in which to study the genomics of host specialization and coevolution. Specialist *Metarhizium*

strains and species generally have fewer genes, with notably fewer genes encoding host-killing toxins (Wang et al. 2009), but more genes evolving under positive selection than generalists (Hu et al. 2014). However, it remains unclear how adaptation has shaped the genomes of closely related fungal entomopathogens specializing on different hosts.

We therefore tried to identify genes involved in host specificity in three complexes of closely related species from the genus *Ophiocordyceps* (Hypocreales, Ascomycota): *O. unilateralis sensu lato*, *O. subramanianii s.l.* and *O. australis s. l.* One of the key features of these pathogens is their ability to manipulate their hosts to promote their own dispersal. Infected ants, often described as “zombie ants”, leave their nests and develop erratic behavior, wandering alone into vegetation and then biting into a leaf located at a precise height and orientation optimal for subsequent fungal dispersal just before they die. Fungal spores produced from the diseased ant are thus dispersed farther, from a height (Hughes et al. 2011; Pontoppidan et al. 2009; De Bekker et al. 2017; Hughes et al. 2016). *Ophiocordyceps unilateralis s.l.* is a highly diverse complex of pathogenic cryptic species specific to formicine ants. It is distributed worldwide and many species occur together in sympatry while displaying strong host specificity (Araújo et al. 2017, Evans et al. 2011; Kobmoo et al. 2012, 2015). Ants develop erratic behavior only when infected with their specific pathogen species (De Bekker et al. 2014, Sakolrak et al. 2018). The taxonomy and phylogeny of the other ant-manipulating *Ophiocordyceps* species complexes have been studied in less detail, but host specificity is also considered to be the rule for these other taxa (Araujo et al. 2017).

We conducted a comparative genomic study of ant-infecting *Ophiocordyceps* species, with the aim of identifying genes underlying host specificity by searching for species-specific genes and genes evolving under positive selection. We sequenced the genomes of two closely related species

of the *O. unilateralis* complex from Thailand: *O. camponoti-leonardi* and *O. camponoti-saundersi*, specific to the ants *Camponotus leonardi* and *C. saundersi*, respectively. We also improved the available genome assembly of another species of this complex, *O. polyrhachis-furcata*, specific to *Polyrhachis furcata* (Wichadakul et al. 2015), and used the published genomes of other ant-infecting *Ophiocordyceps* species (De Bekker et al. 2017): one genome of each of two species of *O. unilateralis* s.l., *O. kimflemingiae* from the US infecting *C. castaneus* (Araujo et al. 2017; de Bekker et al. 2015) and *O. camponoti-rufipedis* from Brazil specific to *C. rufipes* (Araujo et al. 2017; Evans et al. 2011); one genome of *O. subramanianii* s.l. from a ponerine ant in Ghana; one genome of each of two strains of *O. australis* s.l. found on different ponerine ant species, from Ghana and Brazil, probably belonging to different cryptic species (De Bekker et al. 2017).

The entomopathogenic fungi of the order Hypocreales are known to infect their host by penetrating the cuticle (Boomsma et al. 2014). This process requires an array of proteinases, lipases and chitinases. The acquisition of nutrients from the host requires proteases and glycoside hydrolases, including trehalases in particular, as trehalose is a major carbon source present in the insect hemolymph (Thompson 2003). Secondary metabolites, including toxins, help to combat the host immune system and eventually kill the insect (Ortiz-Urquiza et al. 2010; Schrank and Vainstein 2010). *Ophiocordyceps polyrhachis-furcata* has a more extensive family of genes encoding putative heat-labile enterotoxins than other specialist entomopathogenic fungi (Wichadakul et al. 2015), and some of these genes are expressed during host-specific behavioral manipulation. Heat-labile enterotoxins may, therefore, act as neuromodulators (de Bekker et al. 2015). We hypothesized that enterotoxin-coding genes would be under recurrent positive selection in ant-manipulating *Ophiocordyceps* fungi, as they have probably been involved in coevolution with the host and in host-specific adaptation. Small proteins secreted by fungal pathogens are often

involved in interactions with the host (Barrett and Heil 2012; Rafiqi et al. 2012). We therefore conducted genome scans for positive selection and focused on the heat-labile enterotoxin gene family and small secreted proteins. We conducted formal tests for positive selection (statistical comparisons of models of evolution with and without diversifying selection). As such tests detect only highly recurrent and rapid positive selection, we also investigated the 5% of genes with the highest dN/dS values. High dN/dS values, even if below 1, may be indicative of positive selection at a few sites in the protein, although they may also result from relaxed selection. In several classes of genes thought to be important for virulence and pathogenicity in entomopathogenic fungi (e.g., chitinases, lipases, proteases, small secreted proteins), only a few genes showed signs of selection or species specificity. By contrast, we found that heat-labile enterotoxins were overrepresented among both the species-specific genes and the genes with significant footprints of positive selection. An analysis including enterotoxin-encoding genes from other entomopathogenic fungi (Hypocreales), that do not manipulate host behavior, showed that positive selection was specific to the ant-infecting genus *Ophiocordyceps*. These findings suggest that heat-labile enterotoxins are important effectors involved in host adaptation and coevolution in entomopathogenic *Ophiocordyceps* fungi.

Materials and methods

Sampling and sequencing

In 2015, we collected a sample of *O. camponoti-leonardi* (strain NK511ss-8) from Kalayaniwattana district, in Chiang Mai province in Thailand, and a sample of *O. camponoti-saundersi* (strain NK405ss-6) from the Phu Kiew National Park, in Chaiyaphum province. We used

the reference genome of *O. polyrhachis-furcata* (strain BCC54312) (Wichadakul et al. 2015); we aimed to improve the existing reference genome, but the original strain BCC54312 could not be grown from the culture collection. We therefore collected three additional samples of this species (strains NK275ss-12, NK142ss and NK294ss-20), in 2013 and 2014, from the same site as the reference strain, in Khao Yai National Park, Nakhon Rachasima province. The collected samples were isolated and grown as described by Wongsas et al. (2005). We complied with the Nagoya protocols on access and benefit-sharing, by obtaining authorization from the Department of National Parks, Wildlife and Plant Conservation (DNP) at the Ministry of Natural Resources and Environment of Thailand for all strain collections. After two to three months of growth on Grace Insect Cell Medium (Sigma-Aldrich), the mycelia and spores were harvested and DNA was extracted with the NucleoSpin® Soil kit (Macherey-Nagel). The long incubation period is due to the fact that *O. unilateralis* species in Thailand are very fastidious to grow, requiring few steps of enlarging the culture scale to a sufficient level for DNA extraction. Genomic libraries were constructed (150 bp paired-end reads) for sequencing with an Illumina HiSeq3000 machine at the GenoToul platform (Toulouse, France).

Read pretreatment, de novo assembly and improvement of the reference genome

The raw reads were trimmed to remove adapters and low-quality bases from their ends ($q > 20$). Duplicate reads were removed using Picard Tools MarkDuplicate. The reference genomes for *O. camponoti-leonardi* and *O. camponoti-saundersi* were assembled *de novo* with SPAdes (Bankevich et al. 2012), which progressively integrates k-mers of increasing size. The k-mer sizes used were 21, 33, 55, 77, 99, 119, and 127 for NK405ss-6, and 21, 33, 55, 77, 99 and 115 for

NK511ss-8. The appropriate maximum k-mer sizes were estimated with Kmergenie (Chikhi and Medvedev 2013).

The reads obtained for the new *O. polyrhachis-furcata* samples were used to fill gaps in the existing reference genome of this species with GapFiller (Boetzer et al. 2012), which mapped the reads onto the reference sequence over the regions flanking the gaps and identified a consensus between reads overlapping the gaps. In total, 175 of 3915 gaps were closed (identifying around 1.6 Mb from a total gap length of 2.4 Mb in the reference genome).

Gene prediction and functional annotation

Gene prediction was based exclusively on scaffolds of more than 1 kb in length, and involved a two-round approach based on MAKER (Cantarel et al. 2008). Gene sets were initially predicted with CEGMA (Parra et al. 2007) and GeneMark-ES (Lomsadze et al. 2005), and were then used as inputs into MAKER for the first round of prediction. The predicted proteins and transcripts identified in previous studies on *O. polyrhachis-furcata* (Wichadakul et al. 2015) were also used as a training set for MAKER. The predicted gene set from this first round was then fed into SNAP (Korf 2004) and Augustus (Keller et al. 2011). The output of these two tools was then fed back into MAKER for a second round of prediction.

The predicted proteins were annotated with InterProScan 5 (Jones et al. 2014), which also associated the protein domains detected with sequences in the Pfam (Finn et al. 2016) and KEGG (Ogata et al. 1999; Kanehisa et al. 2016) databases and with Gene Ontology (GO) terms. Small secreted proteins (SSPs) were identified as proteins of less than 300 amino acids with signal peptides but no transmembrane signature detected by SignalP (Petersen et al. 2011). Proteolytic enzymes were also annotated, by Blast analysis of the predicted proteins against the MEROPS

database (Rawlings et al. 2016) with a threshold e-value of $1e^{-20}$. Enzymes with activity against carbohydrates were annotated with dbCAN (Yin et al. 2012); only genes with Pfam domains consistent with those of the CAZYme database were retained. InterProScan recognized glycoside hydrolase domains for some genes that were not detected by the CAZYme analysis toolkit; these genes were also retained. The core genes of secondary metabolic gene clusters (SMGCs) were predicted with SMURF (Khaldi et al. 2010), based on Pfam and Tigrfam domains and on gene positions on scaffolds. SMGCs were predicted with the fungal version of antiSMASH (Weber et al. 2015). SMGC homology across species was inferred with BiG-SCAPE (Navarros-Munz J., <https://git.wageningenur.nl/medema-group/BiG-SCAPE/wikis/home>), which classified SMGCs into families on the basis of Jaccard similarity indices between clusters. RepeatMasker was used to predict repetitive elements for the three species from Thailand.

Orthology and phylogenomics

In addition to the predicted proteins from the *de novo* assembled and improved genomes of *O. unilateralis* species from Thailand, we also included in our analyses the predicted proteins of other ant-infecting *Ophiocordyceps* fungi specific to different ant species and originating from different geographic areas (De Bekker et al. 2017). We used the available genomes from two additional *O. unilateralis s.l.* species (*O. kimflemingiae* from the USA and *O. camponoti-rufipedis* from Brazil), from two cryptic species of *O. australis s.l.*, from Ghana and Brazil (De Bekker et al. 2017), and from *O. subramanianii s.l.*, also from Ghana. The predicted proteins corresponding to all these genomes were subjected to Blast comparisons with each other, with a significance threshold e-value of $1e^{-5}$. The Blast results were used as input for orthAgogue (Ekseth et al. 2014), a tool for the rapid inference of orthologous groups with the Markov clustering algorithm (MCL,

Dongen 2000). This algorithm recovers species-specific paralogous groups, with genes from a given species considered to be more closely related to each other than to any other gene in any other species. The functional annotations obtained for *O. polyrhachis-furcata* were transferred to the other species for gene copies in the same orthologous group. Species-specific paralogous genes were annotated as described above. We analyzed GO term enrichment among species- or complex-specific paralogs, with the topGO package in R (Alexa and Rahnenfuhrer 2016). Fisher's exact tests were used to compare gene counts between paralogous species-specific or complex-specific groups and the whole gene set for the species or complex, respectively.

Sequences within all orthologous groups were aligned with MACSE (Ranwez et al. 2011) for further analyses. A phylogenetic tree with bootstrap support was constructed according to the GTRCAT model under RaxML-HPC v8.1.5 (Stamatakis 2014), exclusively with the nucleotide sequences for all the one-to-one orthologous groups in which each species was represented.

Detection of positive selection

Pairwise ratios of non-synonymous-to-synonymous substitutions (dN/dS) were calculated between species (Yang and Nielsen 2000) with the yn00 program implemented in PAML v.4.8a (Yang 2007). The mean pairwise dN/dS ratios were calculated and used to assess variation across single-copy orthologous groups with at least 4 species represented. Groups with dS < 0.01, potentially resulting in inaccurate dN/dS estimates, and groups with excessively high dN/dS (>10) ratios, were discarded. The functions overrepresented among the 5% of genes with the highest dN/dS ratios were inferred by an analysis of enrichment in GO terms. A mean dN/dS>1 for a given gene indicates positive selection, whereas high dN/dS values below 1 can be due to positive selection on a small number of sites within the protein or to relaxed selection.

We also formally tested for positive selection by performing site-model likelihood ratio tests (LRTs) with the CodeML program implemented in PAML v.4.8a (Yang 2007), excluding gaps and ambiguous sites and using trees inferred under GTRCAT model from respective orthologous groups. CodeML estimates the parameter omega ($\omega = dN/dS$) by maximum likelihood methods, allowing variation between sites. While the pairwise measures above only approximate synonymous and non-synonymous rates, likelihood ratio tests (LRTs) statistically compare two models of evolution, one in which $\omega < 1$ (null model) at all sites and another in which $\omega > 1$ at some sites (alternative hypothesis of positive selection); LRTs thus indicate whether a model with positive selection is more likely than a model without positive selection. We compared the M7 (beta distribution of ω) and M8 (beta distribution of ω with a proportion of sites with $\omega > 1$; Nielsen & Yang 1998, Yang et al. 2000) models, and the M8a (similar to M8 but with a category of sites evolving with $\omega = 1$) and M8 (Swanson et al. 2003) models in LRTs. Only genes with a p -value below 0.05 after false-discovery rate (fdr) correction were considered significant. The M7 vs. M8 test is known to lack robustness when the probability mass is located around $\omega=1$, in which case this test gives a high proportion of false positives; under these conditions, the M8a vs. M8 test is preferred (Swanson et al. 2003). We ensured the robustness of our results by considering only genes in which significant evolution under positive selection was detected in both tests. We checked for enrichment in particular GO terms among the genes evolving under positive selection.

We also investigated whether genes encoding heat-labile enterotoxins evolved under positive selection specifically in ant-infecting *Ophiocordyceps* and not in other Hypocrealean fungi. We therefore downloaded predicted gene sequences from other Hypocrealean fungi that were annotated as putative heat-labile enterotoxins from the Ensembl Genome database (Herrero et al. 2016). Putative heat-labile enterotoxin genes were retrieved for 14 entomopathogenic fungi

(one strain per species) (Additional Table S1): *Metarhizium anisopliae* ARSEF23 (24 genes), *M. acridum* CQMa 102 (3 genes) (Pattemore et al. 2014); *M. album* ARSEF1941 (12 genes), *M. brunneum* ARSEF3297 (32 genes), *M. guizhouense* ARSEF977 (32 genes), *M. majus* ARSEF297 (32 genes) (Hu et al. 2014); *M. rileyi* RCEF4871 (3 genes), *Isaria fumosorosea* ARSEF2679 (5 genes), *Aschersonia aleyrodis* RCEF2490 (14 genes), *Cordyceps confragosa* RCEF1005 (6 genes), *C. brongniartii* RCEF3172 (30 genes) (Shang et al. 2016), *Cordyceps militaris* CM01 (one gene, Zheng et al. 2011), *Beauveria bassiana* ARSEF2860 (6 genes, Xiao et al. 2012); and *Ophiocordyceps sinensis* Co18 (13 genes, Xia et al. 2017). We also included putative heat-labile enterotoxin sequences from two nematode-killing fungi: *Purpureocillium lilacinum* PLBJ-1 (two genes, Wang et al. 2016) and *Pochonia chlamydosporia* 170 (four genes). Orthologs between these sequences and the putative enterotoxins of *O. unilatealis* species studied here were identified. The occurrence of clade-specific positive selection in *O. unilateralis* was assessed with branch model LRTs in PAML (Yang 1998; Yang and Nielsen 1998) and with the BUSTED test, an alignment-wide test of episodic positive selection (Murrell et al. 2015). Both these tests are log-likelihood ratio tests comparing a model in which positive selection is allowed in the foreground branches (i.e. the clade of interest) to the null model in which positive selection is not allowed. The branch model (Yang and Nielsen 1998), as implemented in PALM, detects positive selection by allowing a candidate clade to have a dN/dS ratio higher than those of the other branches (background branches) without taking into account variation between sites or allowing variation between branches of the same category. By contrast, BUSTED is a stochastic test using information from all sites and branches; it is therefore considered to have greater statistical power (Murrell et al. 2015).

Results

General genome features

The three reference genomes of closely related species sequenced here differed considerably in size, *O. camponoti-saundersi* (OCS) being the largest (49.26 Mb), followed by *O. polyrhachis-furcata* (OPF) (43.25 Mb) and *O. camponoti-leonardi* (OCL) (37.91 Mb). These differences probably partly reflect methodological differences as the OPF genome is an improved version of a genome sequenced with a different technology (454 pyrosequencing combined with Illumina mate-pair sequencing, Wichadakul et al. 2015). OCL and OCS were sequenced and assembled with the same methodology, so the observed differences probably reflect genuine differences in genome size. OCS also had more scaffolds (1700) than OCL (531). OPF had fewer scaffolds and larger contigs, due to the use of variable-size mate-pair libraries (Wichadakul et al. 2015) (Table 1). These genomes are markedly larger than those reported for *O. kimflemingiae* (OKi: 23.91 Mb), *O. camponoti-rufipedis* (OCR: 21.91 Mb), *O. australis s.l.* from Brazil (OAB: 23.32 Mb) and from Ghana (OAG: 22.19 Mb), and *O. subramanianii s.l.* (OSS: 32.31 Mb), but all these previously published genomes were more fragmented than our assemblies (Table 1).

Despite the differences in genome size, the numbers of predicted genes were of a same order of magnitude across species (Table 1), although the number of predicted genes was nevertheless largest for OSS. For the three species from Thailand, OPF had the largest number of predicted genes, probably because the protein and transcript training set used for prediction came from this species. The number of SSPs was similar between the three Thai species. The number of genes with assigned Pfam domains or InterPro classification and the complete predicted gene sets

obtained by core eukaryotic genes mapping (CEGMA) were also very similar in the three species (~95 %: Table 1), but smaller than those for species from the New World (~99 %).

Orthology and phylogenomics

The genomes used in this study were sequenced from individuals belonging to one of the three species complexes: *O. unilateralis s.l.*, *O. australis s.l.* and *O. subramanianii s.l.* Most of the genes were common to all three complexes (Fig. 1a): 8,554 orthologous groups were retrieved, 5,718 of which were common to all complexes. For orthologous groups present in only one of the three complexes (Supporting information: Table S2), pathogenesis (GO:0009405) was the function displaying the most significant enrichment in all complexes (Bonferroni-corrected p -values: $2e^{-10}$ for *O. unilateralis s.l.*, 0.016 for *O. australis s.l.*, $3.4e^{-5}$ for *O. subramanianii s.l.*), mostly due to the presence of genes encoding putative heat-labile enterotoxins in these complex-specific genes. Complex-specific genes were also found to be enriched in inter-species interactions and multi-organism process functions.

Within each species complex, most of the genes were common to several species (Fig. 1b and 1c). The function pathogenesis was found to be overrepresented among species-specific genes (Supporting information: Tables S3 - S4), due to the presence of genes encoding heat-labile enterotoxins, and SSPs (Tables 2 and 3). In particular, we detected an overrepresentation of SSPs among the genes unique to *O. kimflemingiae* (p -value = 0.003) relative to *O. unilateralis s.l.* complex, and among the genes unique to *O. australis* from Brazil relative to *O. australis s.l.* complex (p -value = 0.001). None of these species-specific SSPs had a predicted function, suggesting an expansion of rapidly evolving families of effectors (Kim et al. 2016).

There were 4,651 orthologous groups common to all eight genomes. We used a subset of 4,014 single-copy orthologous groups common to all species to construct a phylogenetic tree (Fig. 2). This tree recovered the expected relationships between the sibling species from Thailand, with *O. polyrhachis-furcata* being the most closely related to *O. camponoti-leonardi* (Kobmoo et al. 2012, 2015); the species from the Americas, *O. kimflemingiae* and *O. camponoti-rufipedis*, clustered together but were separate from those from Thailand, corresponding to the separation between the Old and New Worlds observed in a previous study (Evans et al. 2018). The two *O. australis s.l.* species were grouped together and formed, with *O. subramanianii*, an outgroup to the *O. unilateralis* complex.

Variation of dN/dS across genomes and putative functions

The median pairwise dN/dS ratio was 0.081, indicating that most single-copy orthologs evolved under strong purifying selection (Fig. 3a). No orthologous group had dN/dS > 1 (Supporting information: Table S5). We investigated the putative functions of the 5% of genes with the highest dN/dS values (297 genes) (Supporting information: Table S5), even if these ratios were below 1, as this could be indicative of positive selection at a small number of sites in the protein, although relaxed selection cannot be excluded for dN/dS < 1. Fisher's exact tests for enrichment in GO term annotation among the 5% of genes with the highest dN/dS values relative to all single-copy orthologous groups (i.e., dN/dS > 0.253) indicated that the functions pathogenesis, interspecies interaction between organisms and multi-organism process (respective Bonferroni-corrected *p*-value = 3.4e-17, 2.2e-18 and 5.5e-180.005) were the most significantly overrepresented. The genes annotated with the pathogenesis (GO:0009405) GO term and with a dN/dS ratio in the top 5% were all putative heat-labile enterotoxin alpha chains. Twenty

orthologous groups annotated as heat-labile enterotoxins were found in one-to-one orthologous groups common to at least four species, thirteen of which had dN/dS values in the top 5%. Enterotoxins had a significantly higher mean dN/dS than the genome as a whole (t -test: Bonferroni-corrected p -value = 5.19×10^{-8}) (Fig. 3b). The GO terms relating to glycoside hydrolases (GH families: chitinases GH18, trehalases GH37), proteases and lipases, essential to the primary functions of entomopathogenic fungi, such as nutrient acquisition and host cell penetration, were not overrepresented among the 5% of genes with the highest dN/dS values. The mean dN/dS values for these gene families were not significantly higher than that for the whole genome (t -tests; chitinases: bonferroni-corrected p -value=0.926, lipases: bonferroni-corrected p -value=1.00, proteases: bonferroni-corrected p -value = 1.00, trehalases: bonferroni-corrected p -value=1.00) (Fig. 3b). None of the core genes in secondary metabolic gene clusters was found among the 5% of genes with the highest dN/dS values, and their mean dN/dS value was not significantly higher than that of other genes (t -test: bonferroni-corrected p -value=0.885) (Fig. 3b). The genes encoding putative SSPs had a significantly higher dN/dS ratio than non-SSP genes (t -test: p -value= 2.2×10^{-16}) (Fig. 3b). This suggest that a higher proportion of genes may evolve under positive selection among SSP-encoding genes than among other genes.

Formal tests for positive selection

LRTs (likelihood ratio tests comparing models with or without positive selection) were performed as formal genome-wide tests for positive selection. Of the 5,950 single-copy orthologous groups common to at least four species, 759 and 244 (12.76% and 4.10 %) were found to be evolving under positive selection in comparisons of the models M7 vs. M8 and M8a vs. M8, respectively (Supporting information: Table S6), with 144 genes found to be evolving under

positive selection in both tests. The functions of pathogenesis and toxin activity were significantly overrepresented among the annotations for these 144 genes (Table 4). This enrichment in the pathogenesis and toxin activity functions was due entirely to enterotoxin genes. Five to seven (25% - 35%) putative heat-labile enterotoxin-coding genes were identified as evolving under positive selection, depending on the evolution model tested (Fig. 4 and Supporting information: Table S7). These genes were also predicted to have a function in the extracellular region (GO:0005576), and most were predicted to have signal peptides. Enrichment was also observed for functions relating to kinases (e.g. ATP binding, kinase activity, transferase activities: Table 4.); significant results were obtained for 15 to 31 (8.1% - 16.8 %) kinases in LRTs and eight kinases gave significant results in both tests (Fig. 4 and Table S7). Kinases catalyze the transfer of a phosphate group from a high-energy molecule (ATP) to a substrate and are involved in various cellular processes. The proportion of kinases evolving under positive selection was lower than that of heat-labile enterotoxins (Fig. 4), but the numbers of kinases and heat-labile enterotoxins evolving under positive selection were similar, and these two functions were overrepresented among the genes evolving under positive selection. Most of these kinases were annotated as protein kinases, histidine kinases and phosphatidylinositol 3 and 4-kinases. These families of kinases are well known to be involved in cell signaling, essential for pathogen growth and survival and, thus, for pathogenesis and virulence (Lee et al. 2016).

The functions relating to hydrolytic enzymes important for pathogenesis (glycoside hydrolases, lipases, proteases) were not overrepresented among the genes evolving under positive selection. Indeed, the proportions of genes in these families found to be under positive selection were markedly smaller than those for heat-labile enterotoxins (Fig. 4). Nevertheless, several of the genes from these gene families were found to evolve under positive selection in model tests

(Supporting information: Table S7) and can be considered good candidates for involvement in coevolution and host specificity. One to three of the 11 chitinases (GH18) presented significant footprints of positive selection depending on the evolution model considered, and significant *P*-values were obtained in all tests for one of these enzymes. Chitinases are involved in the degradation of the insect cuticle, a major component of the insect exoskeleton, and in the degradation and remodeling of fungal cell walls (Adams 2004; Langner and Göhre 2016). Other GH families converging to various functions such as cellulase, glucanase, glucosidase, galactosidase (e.g., GH5, GH16, GH47, GH76) also included a few genes displaying significant tests of positive selection (Supporting information: Table S7). Neither of the two trehalases (GH37), which are thought to play important roles in nutrient acquisition within the host body, displayed significant signs of positive selection.

Zero to four of the nine subtilisin-like (MEROPS family S08 and S53) and trypsin-like proteases (MEROPS family S01), which are considered to act as cuticle-degrading proteases, presented significant footprints of positive selection, depending on the evolution model considered. Zero to one of 16 putative aspartyl proteases (MEROPS family A01) was found to evolve under positive selection following different models. However, none of these proteases yielded significant results in both tests (Fig. 4). Two to six of the 39 putative lipases yielded significant *P*-values in positive selection tests, and only one yielded significant *P*-values in both tests (Fig. 4; Supporting information: Table S7).

One to four of the seven core genes of secondary metabolites displayed significant signatures of positive selection, depending on the evolution model considered (Supporting information: Table S7). The only gene to yield significant *P*-values in both tests (orthologous group ORTHAg2248, Supporting information Table S7) encoded a polyketide synthase (PKS)-like

protein with a beta-ketoacyl synthase domain. Beta-ketoacyl synthase is involved in fatty-acid biosynthesis and has been shown to be involved in the production of polyketide antibiotics in fungi (Beck et al. 1990). The gene encoding this enzyme is part of a secondary metabolic gene cluster that is highly syntenic across the species of the *O. unilateralis* complex, but located in different clusters in *O. australis* and in *O. subramanianii* (Fig. 5).

We also investigated whether the genes previously identified as encoding possible “neuromodulators” (de Bekker et al. 2017), based on their overexpression during the manipulation of ant behavior, showed signs of positive selection. In total, 12 to 41 of these genes yielded significant results in tests for positive selection (Supporting information: Table S8). Five genes yielded significant results in both tests. These genes encoded a short-chain dehydrogenase, a DNA mismatch repair protein (MutC), a DNA replication factor, an ATPase and a protein with no functional annotation. Seven other genes yielded results only in the M8a vs. M8 test, which is more robust than the M7 vs. M8 test. These seven genes included oxidoreductases clearly involved in metabolic reactions: a protein with a ferric reductase transmembrane-like domain, a flavodoxin oxidoreductase, and an oxidoreductase binding to a molybdopterin cofactor with a cytochrome b5-like heme/steroid-binding domain. The first of these genes was shown to be associated with iron uptake in yeast (Roman et al. 1993), whereas the product of the second mediates iron-free electron transfer. The third of these genes may encode a nitrate reductase or sulfite oxidase, both of which are involved in nitrogen assimilation. These putative neuromodulators thus seem to be involved in host resource utilization. The neurological disorder displayed by zombie ants infected with *Ophiocordyceps* may result from the pathogen outcompeting the host for iron and nitrogen.

Positive selection of heat-labile enterotoxin genes specific to the ant-manipulating O. unilateralis species complex

The above results and those of previous studies (de Bekker et al. 2015; Wichadakul et al. 2015) suggest that heat-labile enterotoxin genes are candidate genes for host-specific adaptation. We therefore investigated whether the positive selection detected above was specific to the ant-infecting *Ophiocordyceps* species or general to Hypocrealean entomopathogenic and nematode-killing fungi. Thirty-six orthologous groups of heat-labile enterotoxin genes were inferred for a group of 16 Hypocrealean entomopathogenic and nematode-killing fungi in addition to our eight focal species (Supporting information: Table S1); 22 of these orthologous groups included at least one sequence from the ant-infecting *Ophiocordyceps*, and 10 (42%) of these groups included only sequences from the ant-infecting *Ophiocordyceps* species. We further analyzed the only group (ORTHA_{gEnt13}) common to at least four of the ant-infecting *Ophiocordyceps* species considered and sequences recovered from other species from Hypocreales, for which both site-model LRTs for positive selection were significant. This group included five sequences each from an *O. unilateralis* species. In a maximum likelihood tree, all the *O. unilateralis* sequences were located on the same branch (Fig. 6). The PAML branch-model LRTs indicated that this gene was evolving under positive selection specifically in the *O. unilateralis* clade (p -values < 0.001). The branch at the base and the internal branches of the *O. unilateralis* clade therefore had significantly higher dN/dS ratios than the other branches (Fig. 6). The BUSTED test, which is similar to PAML branch tests but considered more powerful, also gave a significant result (p -value = 6.16e-14).

Discussion

Enterotoxin genes as major candidate genes underlying host adaptation

Comparative genomic studies of closely related species of fungal pathogens have shown that, in general, genes involved in adaptation, particularly those involved in virulence and pathogenicity, are species-specific, highly divergent and/or under diversifying selection, as a result of the arms race between host and pathogen or specialization on new hosts (Ghanbarnia et al. 2015; Huang et al. 2014; Plissonneau et al. 2017; Stukenbrock et al. 2011). We therefore used an evolutionary comparative genomic approach for identifying genes underlying host adaptation in ant-infecting *Ophiocordyceps* from three species complexes (*O. unilateralis* s.l., *O. australis* s.l. and *O. subramanianii* s.l.). Genome comparisons showed that species complex-specific genes were enriched in genes associated with the function pathogenesis which included genes encoding heat-labile enterotoxins. The species-specific genes were also enriched in this function. However, most species-specific genes lacked functional annotation, perhaps due to their rapid evolution as part of the arms race between pathogen and host, resulting in homology no longer being detectable. Most of the small secreted proteins (SSPs), in particular, lacked predicted functions, but these proteins were particularly abundant among the species-specific genes. SSPs may act as effectors in pathogenicity, but the validation of their function requires further studies.

Heat-labile enterotoxin genes were also overrepresented in the orthologous groups with the highest rates of amino-acid differences between species, suggesting the occurrence of diversifying selection, which was confirmed by formal tests comparing models with and without positive selection. Furthermore, in the cases in which orthologs of enterotoxin genes were found in other entomopathogenic fungi, we inferred that positive selection was specific to the ant-infecting *Ophiocordyceps* clade. These findings support the view that heat-labile enterotoxins are effectors involved in host adaptation, as previously suggested based on observations of enterotoxin overexpression during manipulation of the behavior of the diseased ants (de Bekker et al. 2015)

and of the species-specific nature of behavioral manipulation (de Bekker et al. 2014, Sakolrak et al. 2018). The proximal mechanisms via which enterotoxins act during infection and the manipulation of host behavior remain unclear, but it has been suggested that these molecules interfere with the chemical communication of social insects; bacterial enterotoxins have been shown to affect pheromone production in boll weevils (Wiygul and Sikorowski 1986, 1991). Alterations to chemical communication may contribute to the modification of behavior in infected ant hosts.

Minor role of the cuticle in exerting selective pressure leading to diversifying selection

Hypocrealean entomopathogenic fungi are known to infect their insect hosts by penetrating the cuticle from the outside (Boomsma et al. 2014). An array of hydrolytic enzymes, including chitinases, lipases and proteases, is required to break through the insect cuticle (Ortiz-Urquiza and Keyhani 2013). Chitins are major constituents not only of insect cuticles, but also of fungal cell walls (Lagner and Göhre 2016) while lipids are a major component of the epicuticle waxy layer (Jarrold et al. 2007; Pedrini et al. 2013). Proteases are important for the penetration of the cuticle by fungi, and have been shown to be virulence factors for the infection of insect hosts (Shah et al. 2005). Subtilisin proteases have been shown to play a particularly important role in regulating insect host specificity through the differential expressions of specific isoforms (Bye and Charnley 2008; Mondal et al. 2016). We therefore hypothesized that the genes encoding chitinases, proteases and lipases might have evolved under diversifying selection. However, we found footprints of positive selection for only a few of putative genes encoding these enzymes in the ant-infecting *Ophiocordyceps* species. This challenges the widely accepted view that the insect cuticle, as a major barrier to infections, exerts a strong selective pressure on entomopathogenic fungi, leading

to different host ranges (Boomsma et al. 2014; Ortiz-Urquiza and Keyhani 2013; Wang and St. Leger 2005; Wang et al. 2011). Nevertheless, as the fungi in the three ant-infecting complexes considered here are all pathogens of formicine and ponerine ants, our findings do not rule out diversifying selection occurring across larger phylogenetic scales. These enzymes may be highly conserved among pathogens of formicine and ponerine ants, providing a common arsenal for attacking taxonomically related ants. There may also be constraints in the host or the fungus preventing rapid coevolution through changes to these molecules.

Utilization of host resources

Once inside the host, the pathogen requires other hydrolases for carbon assimilation. Efficient nutrient uptake from the host allows optimal proliferation of the fungus within its host, and ultimately leads to insect death (Luo et al. 2014). It has, therefore, been suggested that host resource utilization is crucial for host specificity (Gillespie et al. 2000). Trehalases, in particular, probably play an important role in this respect. Indeed, the fly pathogen *Entomophthora muscae* (Entomophthorales) carries more trehalase-encoding genes in its genome than its close relative, the generalist *Conidiobolus coronatus*, which is a non-obligate pathogen (De Fine Licht et al. 2017). We identified two trehalases with no positive selection signature as conserved across all species. Other glycoside hydrolases and lipases may be involved in breaking down primary carbon sources (Ortiz-Urquiza and Keyhani 2013; Schrank and Vainstein 2010). However, the evidence for positive selection is less robust for these enzymes. Thus, diversifying selection in ant-pathogenic *Ophiocordyceps* fungi probably acts less strongly on the function of carbon assimilation than on enterotoxins. Again, there may be constraints preventing the rapid evolution of host cuticle or fungal hydrolase and lipase functions.

Nitrogen also plays a key role in the proliferation of entomopathogenic fungi (Luo et al. 2014). However, our results suggest that initial nutrient acquisition via proteinases is not under strong diversifying selection. Genes evolving under positive selection were not enriched in functions related to the assimilation of nitrogen or amino-acid synthesis.

In addition to carbon and nitrogen, iron uptake is also crucial for pathogen success (Bairwa et al. 2017, Haas 2012, Sutak et al. 2008). The candidate neuromodulator genes found to be under positive selection included iron-related oxidoreductases. In particular, one of the proteins identified had a ferric-reductase transmembrane domain, and another was a flavodoxin oxidoreductase. Proteins with ferric-reductase transmembrane domains have been shown to be crucial for ferric iron uptake in yeast (Roman et al. 1993), whereas flavodoxin is an iron-free electron-transfer protein facilitating a range of metabolic reactions in the absence of iron. Specialist entomopathogens kill their hosts more slowly than generalists (Boomsma et al. 2014). In such a context, ant-specific *Ophiocordyceps* might be expected to have developed strategies for hijacking resources from the host. The efficient acquisition of iron and an ability to divert its use may be the key to outcompeting the host during infection.

The role of kinases and signal transduction

Kinase enzymes are widely recognized as participating in various cellular processes, crucial to growth and survival (Lee et al. 2016). The genes under positive selection in the ant-infecting *Ophiocordyceps* were enriched in kinase-related functions. Most were clearly related to signal transduction, which plays a crucial role in interactions between hosts and pathogens (Bahia et al. 2018). Pathogens sense and respond to environmental stimuli, including the expression of virulence factor regulatory systems, in the hostile conditions of the host immune system. As extremely

specialized pathogens, ant-infecting *Ophiocordyceps* fungi must fine-tune their responses in the host body.

Importance of lipid metabolism

Many entomopathogenic fungi are also thought to deploy a plethora of metabolites and toxins within the bodies of their hosts (Schrack and Vainstein 2010; Singh et al. 2016). The nature of these molecules probably differs between groups of insect-pathogenic fungi and remains to be precisely determined, but the principal molecules include polyketides (PKs) and non-ribosomal peptides (NRPs) (Gallo et al. 2013). We detected significant footprints of positive selection in some of the core genes of secondary metabolites. The most notable case concerned a PKS-like function involved in lipid biosynthesis. Lipids have been shown to be involved in pathophysiological processes in pathogenic fungi, but the role of the lipid signaling network in host-specific pathogenicity remains to be determined (Singh and Poeta 2011). Kinases are also known to participate in lipid signaling pathways, and the kinases with significant footprints of positive selection identified included phosphatidylinositol 3 and 4-kinase. The phosphorylated form of phosphatidylinositol plays an important role in lipid and cell signaling (Funaki et al. 2000). Lipid metabolism thus seemed to be subject to diversifying selection, although to a lesser extent than heat-labile enterotoxins.

Conclusions

We focused on three ant-infecting species complexes from genus *Ophiocordyceps*, including closely related species. Complex- and species-specific genes were found to be enriched in genes for heat-labile enterotoxins, and this gene family was found to be evolving under positive

selection to a greater extent than other candidate gene families. Our results thus suggest that the specific adaptation and coevolution of specialist species in the ant-infecting *Ophiocordyceps* fungi to their hosts is dependent on selection occurring within the body of the host rather than during cuticle penetration. By contrast, we detected little positive selection on lipases, proteases or chitinases, although we did identify a few interesting candidate genes from these groups. Comparative genomic studies of entomopathogenic fungi remain scarce, and the few studies that have been performed have focused exclusively on species of agricultural or medical interest. The findings of this study improve our understanding of the mechanisms of fungal adaptation to insect hosts, and future studies on fungal pathogens associated with other groups of insects should provide more general insight into the adaptation of entomopathogenic fungi and a more documented comparison with the mechanisms of adaptation in fungal pathogens of plants. The insect innate immune response seems to be much more specific than that in plants, suggesting a certain level of acquired immune response (Cooper and Eleftherianos 2017). Fungal pathogens of insects would be expected to display extensive expansions and contractions of gene families, as observed in plant pathogens, but the target functions may be different. Additional insight gleaned from entomopathogenic fungi would help to improve our general understanding of the mechanisms of adaptive evolution in eukaryotes.

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References

- Adams, D. J. (2004). Fungal cell wall chitinases and glucanases. *Microbiology*, 150(7), 2029–2035. doi:10.1099/mic.0.26980-0
- Aguileta, G., Lengelle, J., Chiapello, H., Giraud, T., Viaud, M., Fournier, E. *et al.* (2012). Genes under positive selection in a model plant pathogenic fungus, *Botrytis*. *Infection, Genetics and Evolution*, 12(5), 987–996. doi:10.1016/j.meegid.2012.02.012
- Aguileta, G., Lengelle, J., Marthey, S., Chiapello, H., Rodolphe, F., Gendrault, A. *et al.* (2010). Finding candidate genes under positive selection in Non-model species: Examples of genes involved in host specialization in pathogens. *Molecular Ecology*, 19(2), 292–306. doi:10.1111/j.1365-294X.2009.04454.x
- Aguileta, G., Refrégier, G., Yockteng, R., Fournier, E., & Giraud, T. (2009). Rapidly evolving genes in pathogens: Methods for detecting positive selection and examples among fungi, bacteria, viruses and protists. *Infection, Genetics and Evolution*, 9(4), 656–670. doi:10.1016/j.meegid.2009.03.010
- Albalat, R., & Cañestro, C. (2016). Evolution by gene loss. *Nature Reviews Genetics*, 17(7), 379–391. doi:10.1038/nrg.2016.39
- Alexa A and Rahnenfuhrer J (2016). *topGO: Enrichment Analysis for Gene Ontology*. R package version 2.28.0.
- Anderson, J. P., Gleason, C. A., Foley, R. C., Thrall, P. H., Burdon, J. B., & Singh, K. B. (2010). Plants versus pathogens: An evolutionary arms race. *Functional Plant Biology*, 37(6), 499–512. doi:10.1071/FP09304
- Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., & Daszak, P. (2004). Emerging infectious diseases of plants: Pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology and Evolution*, 19(10), 535–544. doi:10.1016/j.tree.2004.07.021
- Araújo, J. P. M., Evans, H. C., Kepler, R., Hughes, D. P. (2017). Zombie-ant fungi across continents: 15 new species and new combinations with *Ophiocordyceps*. I. Myrmecophilous hirsutelloid species. *Studies in Mycology*, 90, 119-160.
- Badouin, H., Gladieux, P., Gouzy, J., Siguenza, S., Aguileta, G., Snirc, A., Le Prieur, S., Jeziorski, C., Branca, A., Giraud, T. (2017). Widespread selective sweeps throughout the genome of model plant pathogenic fungi and identification of effector candidates. *Molecular Ecology*, 26, 2041-2062. doi:

10.1111/mec.13976

- Bahia, D., Satoskar, A., Dussurget O. (2018). Cell signalling in host-pathogen interactions: The host point of view. *Frontiers in Immunology*, 9, 1-4. doi: 10.3389/fimmu.2018.00221
- Bairwa, G., Hee Jung W., Kronstad J. (2017). Iron acquisition in fungal pathogens of human. *Metallomics: integrated biometal science*, 9(3), 215-227. doi:10.1039/c6mt00301j
- Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S. *et al.* (2012). SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *Journal of Computational Biology*, 19(5), 455-477. doi:10.1089/cmb.2012.0021
- Baroncelli, R., Amby, D. B., Zapparata, A., Sarrocco, S., Vannacci, G., Le Floch, G. *et al.* (2016). Gene family expansions and contractions are associated with host range in plant pathogens of the genus *Colletotrichum*. *BMC Genomics*, 17(555), 1-17. doi:10.1186/s12864-016-2917-6
- Barrett, L. G., & Heil, M. (2012). Unifying concepts and mechanisms in the specificity of plant-enemy interactions. *Trends in Plant Science*, 17(5), 282-292. doi:10.1016/j.tplants.2012.02.009
- Beck, J., Ripka, S., Siegner, A., Schiltz, E., Schweizer, E. (1990). The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases. *European Journal of Biochemistry*, 192(2), 487-498.
- Boetzer, M., Pirovano, W., Zerbino, D., Birney, E., Simpson, J., Wong, K. *et al.* (2012). Toward almost closed genomes with GapFiller. *Genome Biology*, 13(6), R56. doi:10.1186/gb-2012-13-6-r56
- Boomsma, J. J., Jensen, A. B., Meyling, N. V., & Eilenberg, J. (2014). Evolutionary Interaction Networks of Insect Pathogenic Fungi. *Annu. Rev. Entomol*, 59, 467-85. doi:10.1146/annurev-ento-011613-162054
- Bye, N. J., & Charnley, A. K. (2008). Regulation of cuticle-degrading subtilisin proteases from the entomopathogenic fungi, *Lecanicillium* spp: Implications for host specificity. *Archives of Microbiology*, 189(1), 81-92. doi:10.1007/s00203-007-0296-8
- Cantarel, B. L., Korf, I., Robb, S. M. C., Parra, G., Ross, E., Moore, B. *et al.* (2008). MAKER: An easy-to-use annotation pipeline designed for emerging model organism genomes. *Genome Research*, 18, 188-196. doi:10.1101/gr.6743907
- Chikhi, R., & Medvedev, P. (2014). Informed and automated k-mer size selection for genome assembly. *Bioinformatics*, 30(1), 31-37. doi:10.1093/bioinformatics/btt310
- Cooper, D., & Eleftherianos, I. (2017). Memory and specificity in the insect immune system: Current perspectives and future challenges. *Frontiers in Immunology*, 8(539), 1-6. doi:10.3389/fimmu.2017.00539
- Dongen, S. V. *Graph Clustering by Flow Simulation*. PhD thesis, University of Utrecht, May 2000.
- de Bekker, C., Ohm, R. A., Evans, H. C., Brachmann, A., Hughes, D. P. (2017). Ant-infecting *Ophiocordyceps* genomes reveal a high diversity of potential behavioral manipulation genes and a possible major role for enterotoxins. *Scientific Reports*, 7, 12508. doi:10.1038/s41598-017-12863-w
- de Bekker, C., Ohm, R. A., Loreto, R. G., Sebastian, A., Albert, I., Merrow, M. *et al.* (2015). Gene expression during zombie ant biting behavior reflects the complexity underlying fungal parasitic behavioral manipulation. *BMC Genomics*, 16(1), 1-23. doi:10.1186/s12864-015-1812-x

684 de Bekker, C., Quevillon, L. E., Smith, P. B., Fleming, K. R., Ghosh, D., Patterson, A. D., & Hughes, D. P.
685 (2014). Species-specific ant brain manipulation by a specialized fungal parasite. *BMC Evolutionary*
686 *Biology*, 14(166), doi:10.1186/s12862-014-0166-3. doi:10.1186/s12862-014-0166-3

687 De Fine Licht, H. H., Jensen, A. B., & Eilenberg, J. (2017). Comparative transcriptomics reveal host-specific
688 nucleotide variation in entomophthoralean fungi. *Molecular Ecology*, 26(7), 2092–2110.
689 doi:10.1111/mec.13863

690 Ekseth, O. K., Kuiper, M., & Mironov, V. (2014). OrthAgogue: An agile tool for the rapid prediction of
691 orthology relations. *Bioinformatics*, 30(5), 734–736. doi:10.1093/bioinformatics/btt582

692 Evans, H. C., Elliot, S. L., & Hughes, D. P. (2011). Hidden diversity behind the zombie-ant fungus
693 ophiocordyceps unilateralis: Four new species described from carpenter ants in Minas Gerais, Brazil.
694 *PLoS ONE*. doi:10.1371/journal.pone.0017024

695 Finn, R. D., Coghill, P., Eberhardt, R. Y., Eddy, S. R., Mistry, J., Mitchell, A. L. *et al.* (2016). The Pfam
696 protein families database: Towards a more sustainable future. *Nucleic Acids Research*, 44(D1), D279–
697 D285. doi:10.1093/nar/gkv1344

698 Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., & Gurr, S. J.
699 (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature*, 484(7393), 186–194.
700 doi:10.1038/nature10947

701 Funaki, M., Katagiri, H., Inukai, K., Kikuchi, M., Asano, T. Structure and function of phosphatidylinositol-
702 3,4 kinase. *Cellular Signalling*, 12(3), 135–142. doi:10.1016/S0898-6568(99)00086-8

703 Gallo, A., Ferrara, M., & Perrone, G. (2013). Phylogenetic study of polyketide synthases and nonribosomal
704 peptide synthetases involved in the biosynthesis of mycotoxins. *Toxins*, 5(4), 717–742.
705 doi:10.3390/toxins5040717

706 Gao, Q., Jin, K., Ying, S. H., Zhang, Y., Xiao, G., Shang, Y. *et al.* (2011). Genome sequencing and
707 comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M.*
708 *acridum*. *PLoS Genetics*, 7(1), e1001264. doi:10.1371/journal.pgen.1001264

709 Ghanbarnia, K., Fudal, I., Larkan, N. J., Links, M. G., Balesdent, M. H., Profotova, B. *et al.* (2015). Rapid
710 identification of the *Leptosphaeria maculans* avirulence gene AvrLm2 using an intraspecific
711 comparative genomics approach. *Molecular Plant Pathology*, 16(7), 699–709. doi:10.1111/mpp.12228

712 Gillespie, J. P., Bailey, A. M., Cobb, B., & Vilcinskis, A. (2000). Fungi as elicitors of insect immune
713 responses. *Archives of Insect Biochemistry and Physiology*, 44(2), 49–68. doi:10.1002/1520-
714 6327(200006)44:2<49::AID-ARCH1>3.0.CO;2-F

715 Gladieux, P., Ropars, J., Badouin, H., Branca, A., Aguilera, G., De Vienne, D. M. *et al.* (2014). Fungal
716 evolutionary genomics provides insight into the mechanisms of adaptive divergence in eukaryotes.
717 *Molecular Ecology*, 23(4), 753–773. doi:10.1111/mec.12631

718 Haas, H. (2012). Iron - a key nexus in the virulence of *Aspergillus fumigatus*. *Frontiers in Microbiology*,
719 3(28), 1–10. doi:10.3389/fmicb.2012.00028

720 Hartmann, F. E., Rodríguez de la Vega, R. C., Brandenburg, J. T., Carpentier, F., Giraud, T. (2018) Species-
721 specificity and phylogeographic structure of gene presence-absence polymorphism in castrating
722 anther-smut fungi. *GBE*, *in press*.

723 Herrero, J., Muffato, M., Beal, K., Fitzgerald, S., Gordon, L., Pignatelli, M. *et al.* (2016). Ensembl

comparative genomics resources. *Database: The Journal of Biological Databases and Curation*,
bav096, 1–17. doi:10.1093/database/bav096

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang, X. *et al.* (2014). Trajectory and genomic determinants
of fungal-pathogen speciation and host adaptation. *Proceedings of the National Academy of Sciences
of the United States of America*, 111(47), 1–6. doi:10.1073/pnas.1412662111

Huang, J., Si, W., Deng, Q., Li, P., & Yang, S. (2014). Rapid evolution of avirulence genes in rice blast
fungus *Magnaporthe oryzae*. *BMC Genetics*, 15(45), 1–10. doi:10.1186/1471-2156-15-45

Hughes, D. P., Andersen, S. B., Hywel-Jones, N. L., Himaman, W., Billen, J., & Boomsma, J. J. (2011).
Behavioral mechanisms and morphological symptoms of zombie ants dying from fungal infection.
BMC Ecology, 11(1), 13. doi:10.1186/1472-6785-11-13

Hughes, D. P., Araújo, J. P. M., Loreto, R. G., Quevillon, L., de Bekker, C., Evans, H. C. (2016). From so
simple a beginning: the evolution of behavioral manipulation by fungi. *Advances in Genetics*, 94, 437-
469. doi:[10.1016/bs.adgen.2016.01.004](https://doi.org/10.1016/bs.adgen.2016.01.004)

Ina, Y. (1996). Pattern of synonymous and nonsynonymous substitutions: An indicator of mechanisms of
molecular evolution. *Journal of Genetics*, 75(1), 91. doi:10.1007/BF02931754

Jarrold, S. L., Moore, D., Potter, U., & Charnley, A. K. (2007). The contribution of surface waxes to pre-
penetration growth of an entomopathogenic fungus on host cuticle. *Mycological Research*, 111(2),
240–249. doi:10.1016/j.mycres.2006.10.007

Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W., McAnulla, C. *et al.* (2014). InterProScan 5: Genome-
scale protein function classification. *Bioinformatics*, 30(9), 1236–1240.
doi:10.1093/bioinformatics/btu031

Juárez-Vázquez, A. L., Edirisinghe, J. N., Verduzco-Castro, E. A., Michalska, K., Wu, C., Noda-García, L.
et al. (2017). Evolution of substrate specificity in a retained enzyme driven by gene loss. *eLife*, 6,
e22679. doi:10.7554/eLife.22679

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2016). KEGG as a reference
resource for gene and protein annotation. *Nucleic Acids Research*, 44(D1), D457–D462.
doi:10.1093/nar/gkv1070

Keller, O., Kollmar, M., Stanke, M., & Waack, S. (2011). A novel hybrid gene prediction method employing
protein multiple sequence alignments. *Bioinformatics*, 27(6), 757–763.
doi:10.1093/bioinformatics/btr010

Khaldi, N., Seifuddin, F. T., Turner, G., Haft, D., Nierman, W. C., Wolfe, K. H., Fedorova, N. D. (2010).
SMURF: genomic mapping of fungal secondary metabolite clusters. *Fungal Genetics and Biology*,
47(9), 736–741. doi:10.1016/j.fgb.2010.06.003.

Kim, K.T., Jeon, J., Choi, J., Cheong, K., Song, H., Choi, G. *et al.* (2016) Kingdom-wide analysis of fungal
small secreted proteins (SSPs) reveals their potential role in host association. *Frontiers in Plant
Science*, 7, 186. doi: [10.3389/fpls.2016.00186](https://doi.org/10.3389/fpls.2016.00186).

Kimura, M. (1983). The neutral theory of molecular evolution. Cambridge University Press.

Kobmoo, N., Mongkolsamrit, S., Tasanathai, K., Thanakitpipattana, D., & Luangsa-Ard, J. J. (2012).
Molecular phylogenies reveal host-specific divergence of *Ophiocordyceps unilateralis sensu lato*
following its host ants. *Molecular Ecology*, 21(12), 3022–3031. doi:10.1111/j.1365-

294X.2012.05574.x

- Kobmoo, N., Mongkolsamrit, S., Wutikhun, T., Tasanathai, K., Khonsanit, A., Thanakitpipattana, D., & Luangsa-Ard, J. J. (2015). New species of *Ophiocordyceps unilateralis*, an ubiquitous pathogen of ants from Thailand. *Fungal Biology*, 119(1), 44–52. doi:10.1016/j.funbio.2014.10.008
- Korf, I. (2004). Gene finding in novel genomes. *BMC Bioinformatics*, 5, 59. doi:10.1186/1471-2105-5-59
- Kurtz, J., Schulenburg, H., & Reusch, T. B. (2016). Host–parasite coevolution—Rapid reciprocal adaptation and its genetic basis. *Zoology*, 119, 241–243. doi:10.1016/j.zool.2016.06.011
- Langner, T., & Göhre, V. (2016). Fungal chitinases: function, regulation, and potential roles in plant/pathogen interactions. *Current Genetics*, 62(2), 243–254. doi:10.1007/s00294-015-0530-x
- Lee K.-T., So Y.-S., Yang D. et al. (2016). Systematic functional analysis of kinases in the fungal pathogen *Cryptococcus neoformans*. *Nature Communications*, 7, 12766. doi: 10.1038/ncomms12766
- Lespinet, O., Wolf, Y. I., Koonin, E. V., & Aravind, L. (2002). The role of lineage-specific gene family expansion in the evolution of eukaryotes. *Genome Research*, 12(7), 1048–1059. doi:10.1101/gr.174302
- Lomsadze, A., Ter-Hovhannisyan, V., Chernoff, Y. O., & Borodovsky, M. (2005). Gene identification in novel eukaryotic genomes by self-training algorithm. *Nucleic Acids Research*, 33(20), 6494–6506. doi:10.1093/nar/gki937
- Luo, Z., Qin, Y., Pei, Y., Keyhani, N. O. (2014). Ablation of the *creA* regulator results in amino acid toxicity, temperature sensitivity, pleiotropic effects on cellular development and loss of virulence in the filamentous fungus *Beauveria bassiana*. *Environmental Microbiology*, 16(4), 1122–1136. doi:10.1111/1462-2920.12352
- Möller, M., & Stukenbrock, E. H. (2017). Evolution and genome architecture in fungal plant pathogens. *Nature Reviews Microbiology*. doi:10.1038/nrmicro.2017.76
- Mondal, S., Bakshi, S., Koris, A., & Vatai, G. (2016). Journey of enzymes in entomopathogenic fungi. *Pacific Science Review A: Natural Science and Engineering*, 18(2), 85–99. doi:10.1016/j.psra.2016.10.001
- Murrell, B., Weaver, S., Smith, M. D., Wertheim, J. O., Murrell, S., Aylward, A. et al. (2015). Gene-wide identification of episodic selection. *Molecular Biology and Evolution*, 32(5), 1365–1371. doi:10.1093/molbev/msv035
- Nielsen, R., & Yang, Z. (1998). Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. *Genetics*, 148, 929–936.
- Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., & Kanehisa, M. (1999). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, 28(1), 27–30. doi:10.1093/nar/27.1.29
- Ohno, S. (1970). *Evolution by Gene Duplication*. Springer-Verlag.
- Ortiz-Urquiza, A., & Keyhani, N. O. (2013). Action on the surface: Entomopathogenic fungi versus the insect cuticle. *Insects*, 4(3), 357–374. doi:10.3390/insects4030357
- Ortiz-Urquiza, A., Riveiro-Miranda, L., Santiago-Álvarez, C., & Quesada-Moraga, E. (2010). Insect-toxic secreted proteins and virulence of the entomopathogenic fungus *Beauveria bassiana*. *Journal of Invertebrate Pathology*, 105(3), 270–8. doi:10.1016/j.jip.2010.07.003

802 Parra, G., Bradnam, K., & Korf, I. (2007). CEGMA: A pipeline to accurately annotate core genes in
803 eukaryotic genomes. *Bioinformatics*, 23(9), 1061–1067. doi:10.1093/bioinformatics/btm071

804 Pattemore, J. A., Hane, J. K., Williams, A. H., Wilson, B. Al, Stodart, B. J., & Ash, G. J. (2014). The genome
805 sequence of the biocontrol fungus *Metarhizium anisopliae* and comparative genomics of *Metarhizium*
806 species, *BMC Genomics*, 15(660), 1–15. doi:10.1186/1471-2164-15-660

807 Pedrini, N., Ortiz-Urquiza, A., Huarte-Bonnet, C., Zhang, S., & Keyhani, N. O. (2013). Targeting of insect
808 epicuticular lipids by the entomopathogenic fungus *Beauveria bassiana*: Hydrocarbon oxidation within
809 the context of a host-pathogen interaction. *Frontiers in Microbiology*, 4(24), 1–18.
810 doi:10.3389/fmicb.2013.00024

811 Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides
812 from transmembrane regions. *Nature Methods*, 8(10), 785–786. doi:10.1038/nmeth.1701

813 Plissonneau, C., Benevenuto, J., Mohd-Assaad, N., Fouché, S., Hartmann, F. E., Croll, D. (2017). Using
814 Population and Comparative Genomics to Understand the Genetic Basis of Effector-Driven Fungal
815 Pathogen Evolution. *Frontiers in Plant Science*, 8, 119. doi:[10.3389/fpls.2017.00119](https://doi.org/10.3389/fpls.2017.00119)

816 Pontoppidan, M. B., Himaman, W., Hywel-Jones, N. L., Boomsma, J. J., & Hughes, D. P. (2009).
817 Graveyards on the move: The spatio-temporal distribution of dead ophiocordyceps-infected ants. *PLoS*
818 *ONE*, 4(3), e4835. doi:10.1371/journal.pone.0004835

819 Poppe, S., Dorsheimer, L., Happel, P., Stukenbrock, E. H. (2015). Rapidly Evolving Genes Are Key Players
820 in Host Specialization and Virulence of the Fungal Wheat Pathogen *Zymoseptoria tritici*
821 (*Mycosphaerella graminicola*). *PLoS Pathogens*, 11(7), e1005055.

822 Rafiqi, M., Ellis, J. G., Ludowici, V. A., Hardham, A. R., & Dodds, P. N. (2012). Challenges and progress
823 towards understanding the role of effectors in plant-fungal interactions. *Current Opinion in Plant*
824 *Biology*, 15(4), 477-482. doi:10.1016/j.pbi.2012.05.003

825 Roman D., Dancis A., Anderson G., Klausner R. (1993). The fission yeast ferric reductase gene *frp1+* is
826 required for ferric uptake and encodes a protein that is homologous to the gp91-phox subunit of the
827 human NADPH phagocyte oxidoreductase. *Molecular and Cellular Biology*, 13(7), 4342-4350. doi:
828 10.1128/MCB.13.7.4342

829 Ranwez, V., Harispe, S., Delsuc, F., & Douzery, E. J. P. (2011). MACSE: Multiple alignment of coding
830 SEquences accounting for frameshifts and stop codons. *PLoS ONE*, 6(9), e22594.
831 doi:10.1371/journal.pone.0022594

832 Rawlings, N. D., Barrett, A. J., Finn, R. (2016). Twenty years of the MEROPS database of proteolytic
833 enzymes, their substrates and inhibitors. *Nucleic Acids Research*, 44(D1), D343-D350. doi:
834 10.1093/nar/gkv1118

835 Rouxel, T., & Balesdent, M. H. (2017). Life, death and rebirth of avirulence effectors in a fungal pathogen
836 of Brassica crops, *Leptosphaeria maculans*. *New Phytologist*, 214(2), 526-532.
837 doi:10.1111/nph.14411

838 Sakolrak, B., Blatrix, R., Sangwanit, U., Arnamnart, N., Kobmoo, N. (2018). Experimental infection of the
839 ant *Polyrhachis furcata* with *Ophiocordyceps* reveals specificity of behavioral manipulation. *Fungal*
840 *Ecology*, 33, 122-124. doi: 10.1016/j.funeco.2018.03.001

841 Schirrmann, M. K., Zoller, S., Croll, D., Stukenbrock, E. H., Leuchtman, A., Fior, S. (2018). Genomewide

842 signatures of selection in *Epichloë* reveal candidate genes for host specialization. *Molecular Ecology*,
843 2018 (00), 1–17. doi:[10.1111/mec.14585](https://doi.org/10.1111/mec.14585)

844 Schrank, A., & Vainstein, M. H. (2010). *Metarhizium anisopliae* enzymes and toxins. *Toxicon*, 56(7), 1267–
845 1274. doi:10.1016/j.toxicon.2010.03.008

846 Sexton, A. C., & Howlett, B. J. (2006). Parallels in fungal pathogenesis on plant and animal hosts.
847 *Eukaryotic Cell*, 5(12), 1941–1949. doi:10.1128/EC.00277-06

848 Shah, F. A., Wang, C. S., & Butt, T. M. (2005). Nutrition influences growth and virulence of the insect-
849 pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiology Letters*, 251(2), 259–266.
850 doi:10.1016/j.femsle.2005.08.010

851 Shang, Y., Xiao, G., Zheng, P., Cen, K., Zhan, S., & Wang, C. (2016). Divergent and Convergent Evolution
852 of Fungal Pathogenicity. *Genome Biology and Evolution*, 8(5), 1374–1387. doi:10.1093/gbe/evw082

853 Singh, A., & Poeta, M. D. (2011). Lipid signalling in pathogenic fungi. *Cellular Microbiology*, 13(2), 177–
854 185. doi:10.1111/j.1462-5822.2010.01550.x

855 Singh, D., Son, S. Y., & Lee, C. H. (2016). Perplexing metabolomes in fungal-insect trophic interactions:
856 A Terra incognita of mycobiococontrol mechanisms. *Frontiers in Microbiology*, 7, 1678.
857 doi:10.3389/fmicb.2016.01678

858 Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large
859 phylogenies. *Bioinformatics*, 30(9), 1312–1313. doi:10.1093/bioinformatics/btl446

860 Stukenbrock, E. H., Bataillon, T., Dutheil, J. Y., Hansen, T. T., Li, R., Zala, M. *et al.* (2011). The making
861 of a new pathogen: Insights from comparative population genomics of the domesticated wheat
862 pathogen *Mycosphaerella graminicola* and its wild sister species. *Genome Research*, 21(12), 2157–
863 2166. doi:10.1101/gr.118851.110

864 Sutak R., Lesuisse E., Tacherzy J., Richardson D. (2008) Crusade for iron: iron uptake in unicellular
865 eukaryotes and its significance for virulence. *Trends in Microbiology*, 16(6), 261–268. doi:
866 10.1016/j.tim.2008.03.005

867 Swanson, W. J., Nielsen, R., & Yang, Q. (2003). Pervasive adaptive evolution in mammalian fertilization
868 proteins. *Molecular Biology and Evolution*, 20(1), 18–20.
869 doi:10.1093/oxfordjournals.molbev.a004233

870 Thompson, S. N. (2003). Trehalose - The Insect “Blood” Sugar. *Advances in Insect Physiology*, 31, 205–
871 285. doi:10.1016/S0065-2806(03)31004-5

872 Wang, C., & Feng, M. G. (2014). Advances in fundamental and applied studies in China of fungal biocontrol
873 agents for use against arthropod pests. *Biological Control*, 68(1), 129–135.
874 doi:10.1016/j.biocontrol.2013.06.017

875 Wang, C., & St. Leger, R. J. (2005). Developmental and Transcriptional Responses to Host and Nonhost
876 Cuticles by the Specific Locust Pathogen *Metarhizium anisopliae* var. *acridum*. *Eukaryotic Cell*, 4(5),
877 937–947. doi:10.1128/EC.4.5.937–947.2005

878 Wang, C., & Wang, S. (2017). Insect Pathogenic Fungi: Genomics, Molecular Interactions, and Genetic
879 Improvements. *Annu. Rev. Entomol.*, 62, 73–90. doi:10.1146/annurev-ento-031616-035509

880 Wang, G., Liu, Z., Lin, R., Li, E., Mao, Z., Ling, J. *et al.* (2016). Biosynthesis of Antibiotic Leucinostatins

881 in Bio-control Fungus *Purpureocillium lilacinum* and Their Inhibition on *Phytophthora* Revealed by
882 Genome Mining. *PLoS Pathogens*, 12(7), e1005685. doi:10.1371/journal.ppat.1005685

883 Wang S, Fang W, Wang C, St. Leger RJ (2011) Insertion of an Esterase Gene into a Specific Locust
884 Pathogen (*Metarhizium acridum*) Enables It to Infect Caterpillars. *PLoS Pathog* 7(6): e1002097.
885 doi:10.1371/journal.ppat.1002097

886 Wang, S., Leclercque, A., Pava-Ripoll, M., Fang, W., & St. Leger, R. J. (2009). Comparative genomics using
887 microarrays reveals divergence and loss of virulence-associated genes in host-specific strains of the
888 insect pathogen *metarhizium anisopliae*. *Eukaryotic Cell*, 8(6), 888–898. doi:10.1128/EC.00058-09

889 Wichadakul, D., Kobmoo, N., Ingsriswang, S., Tangphatsornruang, S., Chantasingh, D., Luangsa-Ard, J. J.,
890 & Eurwilaichitr, L. (2015). Insights from the genome of *Ophiocordyceps polyrhachis-furcata* to
891 pathogenicity and host specificity in insect fungi. *BMC Genomics*, 16, 881. doi:10.1186/s12864-015-
892 2101-4

893 Wicker, T., Oberhaensli, S., Parlange, F., Buchmann, J. P., Shatalina, M., Roffler, S. *et al.* (2013). The
894 wheat powdery mildew genome shows the unique evolution of an obligate biotroph. *Nature Genetics*,
895 45(9), 1092–1096. doi:10.1038/ng.2704

896 Wiygul, G., & Sikorowski, P. P. (1986). The effect of staphylococcal enterotoxin B on pheromone
897 production in fat bodies isolated from male boll weevils. *Journal of Invertebrate Pathology*, 47(1),
898 116–119.

899 Wiygul, G., & Sikorowski, P. P. (1991). The effect of a heat-stable enterotoxin isolated from *Escherichia*
900 *coli* on pheromone production in fat bodies isolated from male boll weevils. *Entomologia*
901 *Experimentalis et Applicata*, 60(3), 305–308. doi:10.1111/j.1570-7458.1991.tb01551.x

902 Wongsu, P., Tساناتاي, K., Watts, P., & Hywel-Jones, N. (2005). Isolation and in vitro cultivation of the
903 insect pathogenic fungus *Cordyceps unilateralis*. *Mycological Research*, 109(8), 936–940.
904 doi:10.1017/S0953756205003321

905 Xia, E.-H., Yang, D.-R., Jiang, J.-J., Zhang, Q.-J., Liu, Y., Liu, Y.-L. *et al.* (2017). The caterpillar fungus,
906 *Ophiocordyceps sinensis*, genome provides insights into highland adaptation of fungal pathogenicity.
907 *Scientific Reports*, 7, 1806. doi:10.1038/s41598-017-01869-z

908 Xiao, G., Ying, S., Zheng, P., Wang, Z.-L., Zhang, S., Xie, X.-Q. *et al.* (2012). Genomic perspectives on
909 the evolution of fungal entomopathogenicity in *Beauveria bassiana*. *Scientific Reports*, 2, 483.

910 Yang, Z. (1998). Likelihood ratio tests for detecting positive selection and application to primate lysozyme
911 evolution. *Mol Biol Evol*, 15(5), 568–573. doi:10.1093/oxfordjournals.molbev.a025957

912 Yang, Z. (2007). PAML 4: Phylogenetic analysis by maximum likelihood. *Molecular Biology and*
913 *Evolution*, 24(8), 1586–1591. doi:10.1093/molbev/msm088

914 Yang, Z., & Nielsen, R. (1998). Synonymous and Nonsynonymous Rate Variation in Nuclear Genes of
915 Mammals. *Journal of Molecular Evolution*, 46, 409–418.

916 Yang, Z., & Nielsen, R. (2000). Estimating synonymous and nonsynonymous substitution rates under
917 realistic evolutionary models. *Molecular Biology and Evolution*, 17(1), 32–43.
918 doi:10.1093/oxfordjournals.molbev.a026236

919 Yang, Z., Nielsen, R., Goldman, N., & Krabbe Pedersen, A.-M. (2000). Codon-Substitution Models for
920 Heterogeneous Selection Pressure at Amino Acid Sites. *Genetics*, 155, 431–449.

- Yang, Z., Wong, W. S. W., & Nielsen, R. (2005). Bayes empirical Bayes inference of amino acid sites under positive selection. *Molecular Biology and Evolution*, 22(4), 1107-1118. doi:10.1093/molbev/msi097
- Yin, Y., Mao, X., Yang, J., Chen, X., Mao, F., Xu, Y. (2012). dbCAN: a web resource for automated carbohydrate-active enzyme annotation. *Nucleic Acids Research*, 40(W1), 445-451.
- Zhang, J., Zhang, Y.-P., & Rosenberg, H. F. (2002). Adaptive evolution of a duplicated pancreatic ribonuclease gene in a leaf-eating monkey. *Nature Genetics*, 30, 411–415. doi:10.1038/ng852
- Zheng, P., Xia, Y., Xiao, G., Xiong, C., Hu, X., Zhang, S. *et al.* (2011). Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional chinese medicine. *BMC Genome Biology*, 12(11), R116.
- Zhou, K., Huang, B., Zou, M., Lu, D., He, S., & Wang, G. (2015). Genome-wide identification of lineage-specific genes within *Caenorhabditis elegans*. *Genomics*, 106(4), 242–248. doi:10.1016/j.ygeno.2015.07.002

Data Accessibility

The *de-novo* assemblies of *Ophiocordyceps camponoti-leonardi* (NCBI Biosample SAMN07662903) and *O. camponoti-saundersi* (NCBI Biosample SAMN07662932) have been deposited with the NCBI as whole-genome shotgun (WGS) projects with accession numbers PDHP000000000 and PDHQ000000000, respectively. The *O. polyrhachis-furcata* genome was updated based on the improved assembly from this study (LKCNO20000000).

Author Contributions

N.K., J.J.L. and T.G. designed the study. N.K. and N.A. conducted sampling and DNA extraction. N.K., D.W. and RCRSLV analyzed sequencing and comparative genomic data. N.K. and T.G. wrote the manuscript, with contributions from all the authors.

Supporting information

Table S1. Orthologous clusters of genes encoding heat-labile enterotoxins in species of the Hypocreales.

Table S2. List of genes unique to one of the three complexes: *Ophiocordyceps unilateralis*, *O. australis*, *O. subramanianii*.

Table S3. List of species-specific genes for the *Ophiocordyceps unilateralis* complex.

Table S4. List of species-specific genes for the *Ophiocordyceps australis* complex.

Table S5. List of the 5% of genes with the highest non-synonymous-to-synonymous substitution rate ratios (dN/dS) values, with functional annotations.

Table S6. Complete list of genes with significant likelihood ratio tests (LRTs) after false discovery rate (fdr) correction.

Table S7. Results of likelihood ratio tests (LRTs) for categories of genes thought to be involved in adaptation.

Table S8. Results of likelihood ratio tests (LRTs) for putative neuromodulator genes (after De Bekker et al. 2017).

Figure captions

Figure 1. Inference of orthologous groups: Venn diagram showing the number of orthologous groups common to and specific to species complexes and species a. between the three ant-infecting *Ophiocordyceps* species complexes used in this study; b. between the species in the *O. unilateralis* complex (OPF = *O. polyrhachis-furcata*, OCL = *O. camponoti-leonardi*, OCS = *O. camponoti-*

saundersi, OKi = *O. kimflemingiae*, OCR = *O. camponoti-rufipedis*), b. between the species in the *O. australis* complex (OAG = *O. australis* from Ghana, OAB = *O. australis* from Brazil).

Figure 2. The best maximum-likelihood tree based on 4,014 single-copy orthologous groups with bootstrap supports. The horizontal scale bar represents the branch length based on substitution rates.

Figure 3. Distribution of pairwise non-synonymous-to-synonymous substitution ratios (dN/dS) for the genes in all orthologous groups in the *Ophiocordyceps unilateralis s.l.* species complex. (a) Whole-genome dN/dS distributions, (b) Boxplots of pairwise dN/dS values for the whole genome (small secreted protein-coding genes or SSPs, in blue, versus non-SSPs, in red) and between different categories of genes suspected *a priori* to be involved in pathogenesis and virulence, i.e., with the putative functions of enterotoxins, core proteins of secondary metabolism (SM), lipases, proteases (including subtilisin-like, trypsin and aspartyl proteases) and trehalases. The dotted line represents the mean dN/dS value for the whole genome (0.145).

Figure 4. Percentages of genes in various functional categories for which likelihood ratio tests (LRTs) for positive selection (M7 vs. M8 and M8a vs. M8) yielded significant results (false discovery rate-corrected p -value < 0.05). Proteases = subtilisin, trypsin and aspartyl proteases, SM = core genes of secondary metabolites. The total number of genes in each category is indicated above the bars.

Figure 5. Homology of putative secondary metabolic gene clusters (SMGCs) with the core gene under positive selection according to log likelihood ratio tests (M7 vs. M8 models and M8a vs. M8 models). The dashed lines indicate orthology between the putative polyketide synthase (PKS)-like core gene. The phylogenetic tree was inferred from Jaccard similarity indices between alignments of common gene domains within families. OCS = *Ophiocordyceps camponoti-saundersi*, OCL = *O. camponoti-leonardi*, OPF = *O. polyrhachis-furcata*, OKi = *O. kimflemingiae*, OCR = *O. camponoti-rufipedis*, OAG = *O. australis* from Ghana, OAB = *O. australis* from Brazil, OSS = *O. subramanianii*.

Figure 6. The best RAxML tree based on the GTRCAT model for the orthologous group ORTHAgEnt13 of putative heat-labile enterotoxins in entomopathogenic and nematode-killing fungi of the order Hypocreales. The numbers above the nodes are bootstrap supports. The numbers below the branches are the ratios of non-synonymous-to-synonymous substitution rates (dN/dS).

FIGURE 2 The best maximum-likelihood tree based on 4,014 single-copy orthologous groups with bootstrap supports. The horizontal scale bar represents the branch length based on substitution rates

FIGURE 3 Distribution of pairwise nonsynonymous-to-synonymous substitution ratios (dN/dS) for the genes in all single-copy orthologous groups with at least four species of ant-infecting *Ophiocordyceps* represented. (a) Whole-genome dN/dS distributions, (b) Boxplots of pairwise dN/dS values for the whole genome (small secreted protein-coding genes or SSPs, in blue, vs. non-SSPs, in red) and between different categories of genes suspected a priori to be involved in pathogenesis and virulence, that is, with the putative functions of enterotoxins, core proteins of secondary metabolism (SM), lipases, proteases (including subtilisin-like, trypsin and aspartyl proteases) and trehalases. The dotted line represents the mean dN/dS value for the whole genome (0.145)

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FIGURE 4 Percentages of genes in various functional categories for which likelihood ratio tests (LRTs) for positive selection (M7 vs. M8 and M8a vs. M8) yielded significant results (false-discovery rate-corrected p -value < 0.05).

Proteases = subtilisin, trypsin and aspartyl proteases, SM = core genes of secondary metabolites. The total number of genes in each category is indicated above the bars

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FIGURE 5 Homology of putative secondary metabolic gene clusters (SMGCs) with the core gene under positive selection according to log-likelihood ratio tests (M7 vs. M8 models and M8a vs. M8 models). The dashed lines indicate orthology between the putative polyketide synthase (PKS)-like core gene. The phylogenetic tree was inferred from Jaccard similarity indices between alignments of common gene domains within families. OCS = *Ophiocordyceps camponoti-saundersi*, OCL = *O. camponoti-leonardi*, OPF = *O. polyrhachis-furcata*, OKI = *O. kimflemingiae*, OCR = *O. camponoti-rufipedis*, OAG = *O. australis* from Ghana, OAB = *O. australis* from Brazil, OSS = *O. subramanianii*