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Laterally Transferred Gene Recruited as a Venom in Parasitoid Wasps

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Abstract

Parasitoid wasps use venom to manipulate the immunity and metabolism of their host insects in a variety of ways to provide resources for their offspring. Yet, how genes are recruited and evolve to perform venom functions remain open questions. A recently recognized source of eukaryotic genome innovation is lateral gene transfer (LGT). Glycoside hydrolase family 19 (GH19) chitinases are widespread in bacteria, microsporidia, and plants where they are used in nutrient acquisition or defense, but have previously not been known in metazoans. In this study, a GH19 chitinase LGT is described from the unicellular microsporidia/*Rozella* clade into parasitoid wasps of the superfamily Chalcidoidea, where it has become recruited as a venom protein. The GH19 chitinase is present in 15 species of chalcidoid wasps representing four families, and phylogenetic analysis indicates that it was laterally transferred near or before the origin of Chalcidoidea (~95 Ma). The GH19 chitinase gene is highly expressed in the venom gland of at least seven species, indicating a role in the complex host manipulations performed by parasitoid wasp venom. RNAi knockdown in the model parasitoid *Nasonia vitripennis* reveals that—following envenomation—the GH19 chitinase induces fly hosts to upregulate genes involved in an immune response to fungi. A second, independent LGT of GH19 chitinase from microsporidia into mosquitoes was also found, also supported by phylogenetic reconstructions. Besides these two LGT events, GH19 chitinase is not found in any other sequenced animal genome, or in any fungi outside the microsporidia/*Rozella* clade.

Key words: **lateral gene transfer, parasitoid wasps, venom, microsporidia, mosquito.**

Introduction

Lateral gene transfers (LGTs), the nonsexual transmission of genetic material between distantly related species, are pervasive among bacteria and archaea and are an important source of functional innovation (Doolittle 1999; Ochman et al. 2000; Koonin et al. 2001). Recently, however, genome sequencing has highlighted the unexpected frequency and importance of LGTs in eukaryotic evolution, including both plants and animals (Dunning Hotopp et al. 2007; Dunning Hotopp 2011). LGTs can provide eukaryotes with novel biochemical and other functions not present in their progenitors, for example, the ability to parasitize plants in nematodes (Danchin et al. 2010) and essential amino acid production in sap feeding insects (Husnik et al. 2013; Luan et al. 2015). Therefore, LGTs can be an important source of evolutionary innovation; however, concrete examples of this in the literature are rare. Here, we report an LGT from the unicellular microsporidia/*Rozella* clade into parasitoid wasps of the superfamily Chalcidoidea, where it has become recruited as a venom protein. The vast majority of reported LGTs in insect genomes involve bacterial insertions. To date, only two other examples of a lateral transfer from a eukaryote to an insect have been reported and both involved carotenoid synthesis genes (Moran and Jarvik 2010; Cobbs et al. 2013). The finding presented here therefore doubles the known examples of eukaryotic to insect LGTs, and extends them to both a new protein category (glycoside hydrolases) and a new eukaryote source (microsporidia).

Chalcidoidea (Hymenoptera) is among the most diverse and abundant group of insects on the planet, with 500,000 estimated species (Gibson et al. 1997; Noyes 2003; Heraty 2009). The majority of these wasps are parasitoids of other insects, where females employ venom to induce a variety of metabolic and immunologic manipulations to suppress host immunity and/or provide nutritional resources for their offspring (Rivers and Denlinger 1994; Parkinson et al. 2001; Rivers, Rocco, et al. 2002; Rivers, Ruggiero, et al. 2002; Federici and Bigot 2003; Danneels et al. 2010, 2014; Martinson et al. 2014; Mrinalini et al. 2014). Chalcidoid wasps include both endoparasitoids, which lay eggs within the tissue of their hosts, and ectoparasitoids, which lay eggs on the surface of their hosts (Godfray 1994; Quicke 1997; de Almeida et al. 2002; Rivers, Ruggiero, et al. 2002; Noyes 2003). Chalcidoids have diversified to parasitize over 300 insect families and utilize a range of host developmental stages (e.g., egg, larva, pupa, adult) (Godfray 1994; Quicke 1997). Species within the same genus can display large shifts in host range (e.g., a single species to several orders) (Gibson and Floate

Article

2001; Noyes 2003; Heraty 2009; Desjardins et al. 2010). Because of this ecological diversity, parasitoid venom protein repertoires can be under great pressure to evolve quickly and adopt novel functions. For example, the integration of the entire Polydnaviruses genome has enabled Braconid and Ichneumonid wasps to exploit viral mechanisms for delivering and expressing virulence genes in their hosts (Beck and Strand 2007; Strand 2010; Burke and Strand 2012). Genes acquired through lateral transfers could be a rich source of new functions that may facilitate adaptation to hosts.

Chitin is among the most abundant organic compounds in nature, present in the fungal cell wall and arthropod cuticle (Merzendorfer and Zimoch 2003). Chitinases, which can degrade and alter chitin, are classified into two families of glycoside hydrolases, glycoside hydrolase family 18 (GH18) and glycoside hydrolase family 19 (GH19). GH18 and GH19 chitinases show limited sequence similarity and differ in their catalytic mechanisms and protein structure. GH18 chitinases are the only chitinases found in insects and are widely distributed across the tree of life (Merzendorfer 2013). In contrast, GH19 has a limited and disjunct distribution that does not include Metazoa (see below). The ability to manipulate one of the most abundant polysaccharides in nature endows GH19 chitinases with a valuable function, which can be utilized in different metabolic roles. For example, GH19 chitinases found in plants provide a potent defense against fungi and insects (Fritig et al. 1998; Van Loon et al. 2006), whereas in bacteria GH19 chitinases aid in nutrient and structural molecule acquisition by digesting environmental chitin (Davies and Henrissat 1995; Bhattacharya et al. 2007; Bai et al. 2014). More recently, GH19 chitinase genes have been found in the genomes of microsporidia and *Rozella*, which are unicellular parasites sister to the rest of fungi and commonly infect insects (Roennebaeumer et al. 2006; James et al. 2013). Microsporidial GH19 chitinase expression occurs during infection of the host insect cell (Campbell et al. 2013) and during endospore formation (Roennebaeumer et al. 2006). However, GH19 chitinases have previously not been known to occur in Metazoa.

In this study, we: 1) identify a GH19 chitinase gene of microsporidia/*Rozella* origin that has been laterally transferred into chalcidoid wasps; 2) report its recruitment as a venom in several parasitoid wasp species; 3) use RNA interference knockdown to investigate its effect in envenomated fly hosts; and 4) show a second, independent LGT of GH19 chitinase into a group of mosquitoes.

Results and Discussion

LGT from Microsporidia/*Rozella* into Chalcidoidea

A GH19 chitinase gene from the parasitoid wasp *Nasonia vitripennis* was first identified as a potential LGT because of an atypical orthology pattern (OrthoDB) with the highest protein sequence similarity to microsporidia and an absence in other hymenopteran species (e.g., bee, wasp, ant, or sawfly). Further searches outside of Hymenoptera did not detect GH19 chitinases in any other animal, except a single clade in mosquitoes (see Results below), including absences in all 502 metazoan genomes available on NCBI (accessed December 2014). Remarkably, this GH19 chitinase gene is nearly absent across the greater than 500 My of metazoan evolution (Dunn et al. 2014). In a survey of more recently available chalcidoid genomes and adult female transcriptomes from 12 species of chalcidoid wasps, GH19 chitinase genes are present in 15 of the 16 species surveyed. GH19 chitinase is absent in the fig wasp *Ceratosolen solmsi*, which may be explained by the extreme specialization of its genome as part of an obligate mutualism (Xiao et al. 2013).

The chalcidoid sequences and GH19 chitinase homologs found by searching the NCBI database (see Materials and Methods) were used to construct an amino acid alignment and phylogenetic analysis was preformed with maximum-likelihood and Bayesian methods (fig. 1). Phylogenetic relationships indicate with full posterior probability and bootstrap support that the chalcidoid wasp GH19 chitinase gene was laterally transferred from the microsporidia/*Rozella* clade (fig. 1). The wasp GH19 chitinases are a monophyletic group within microsporidial sequences, indicating a single origin of the LGT. GH19 chitinase's prevalence among species and its presence in the early-branching chalcidoid family Trichogrammatidae, indicate that the LGT occurred near the origin of chalcidoid wasps about 95 Ma (Munro et al. 2011). However, we cannot rule out an earlier transfer occurring prior to the divergence of chalcidoids with Mymarommatidae, Diapriidae, or Cynipoidea wasps, due to the lack of sequenced genomes. All microsporidia (25/25) and the sister taxon *Rozella allomycis* have at least one copy of a GH19 chitinase (Campbell et al. 2013; James et al. 2013). The phylogenetic location of the chalcidoid clade splits the early-branching *Mitosporidium daphniae* from the rest of microsporidia, as well as splitting the microsporidia-*Rozella* sister relationship (fig. 1). The well-supported phylogenetic evidence for an LGT between microsporidia/*Rozella* and chalcidoid wasps is bolstered by the absence of GH19 chitinases from all other metazoans, with the exception of mosquitoes that represents an independent LGT event to be discussed later.

An alternative hypothesis to a LGT of the parasitoid GH19 chitinase could be microsporidial contamination in parasitoid sequencing projects. However, several lines of evidence refute this hypothesis, which we will briefly mention here and discussed more fully below. GH19 chitinases are found on long contigs in all three available chalcidoid wasp genomes (*N. vitripennis*, *Trichogramma pretiosum*, *Copidosoma floridanum*), no other microsporidial genes are present on those contigs, unique introns are present in the wasp GH19 chitinases that are not found in microsporidia, GH19 chitinases are differentially expressed in wasp tissues with no other microsporidial genes present in available genomes, and GH19 chitinase genes are found in numerous chalcidoid species from multiple research groups and using different sequencing methods.

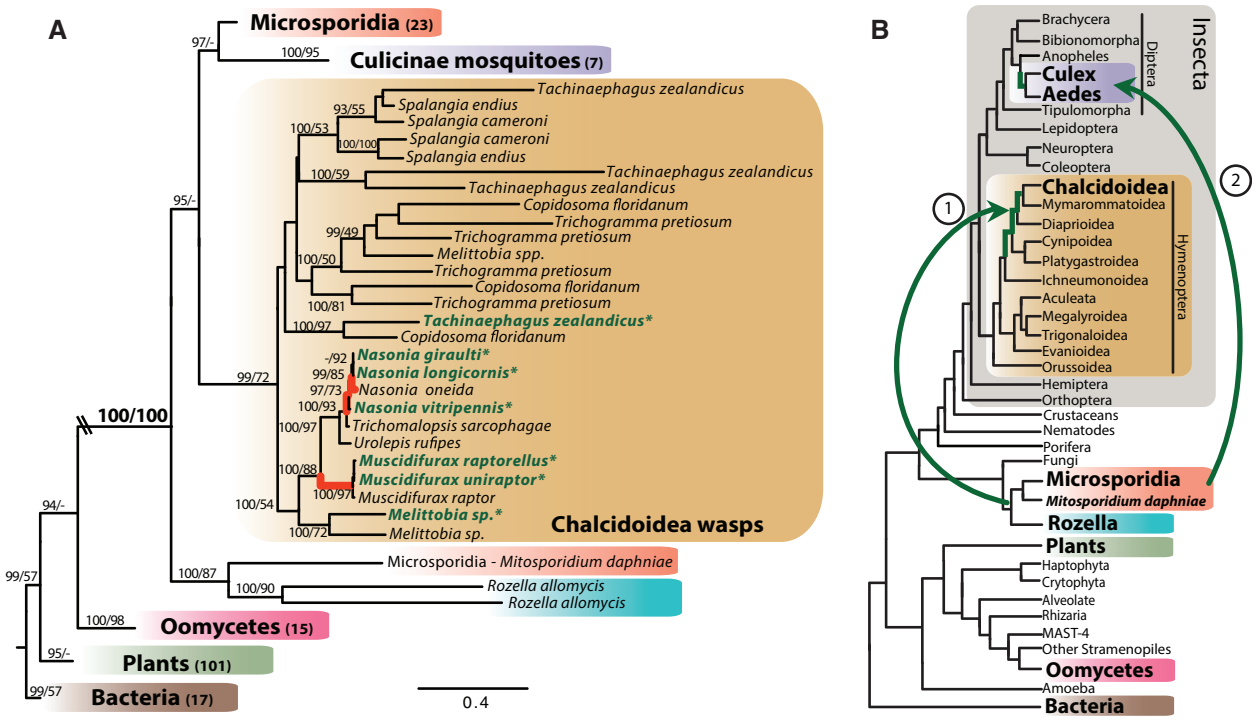

Fig. 1. Phylogeny of GH19 chitinase compared with the tree of life. (*A*) A maximum-likelihood tree of GH19 chitinase protein; nodes labeled with posterior probabilities from a Bayesian analysis (>90) and bootstrap support (>50). Taxa name is followed by the number of sequences in each group. Highlighted taxa with an asterisk indicate GH19 chitinase expression in the venom gland. Bolded branches indicate positive selection (dN/dS ratio > 1). A tree with all sequences and branch support values is available in the supplementary figure S1 (Supplementary Material online). (*B*) The tree of life adapted from previous studies (Wiegmann et al. 2011; Klopfstein et al. 2013; Misof et al. 2014; Peters et al. 2014; Roy et al. 2014). Lines with arrows indicate the direction of horizontal transfers between groups and circled numbers are the proposed chronological order of transfers. Bold and highlighted branches are potential transfer points.

GH19 Chitinase Is a Venom Protein in Many Chalcidoid Wasps

Following transfer, most LGTs are not expressed and are typically expected to degrade by mutation and deletion (Dunning Hotopp et al. 2007). However, some LGTs can evolve into functional genes (Moran and Jarvik 2010; Acuña et al. 2012). To assess whether the GH19 chitinase LGT is expressed in chalcidoids, we surveyed the adult female whole body transcriptomes from 13 species and paired venom gland transcriptomes from 10 species, which were sequenced as part of a larger research effort to understand venom evolution among chalcidoid wasps.

GH19 chitinase genes were detected in the whole body transcriptome of all 13 species and in the venom transcriptomes of seven out of ten species (fig. 2). The average expression of GH19 chitinases in whole body adult females was 323 fragments per kilobase per million (FPKM) (ranging from 1.2 to 3,214 FPKM) (fig. 2). In the venom glands surveyed, seven wasp species (*N. vitripennis*, *N. girault*, *N. longicornis*, *Muscidifurax uniraptor*, *M. raptorellus*, *Melittobia* sp, and *Tachinaephagus zealandicus*) had one copy of the GH19 chitinase gene that, in each case, was highly expressed in the venom gland (average expression of 6,646 FPKM, ranging from 933 to 21,731 FPKM). This indicates that GH19 chitinase plays a role in envenomation (fig. 2). In addition, proteomic screening has found GH19 chitinase peptides in the venom

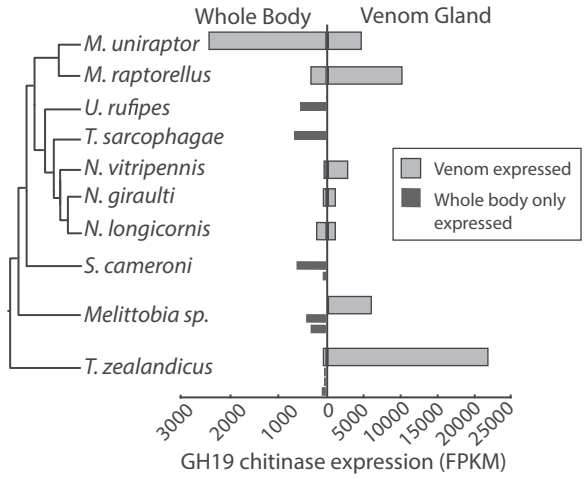

Fig. 2. Expression levels of GH19 chitinase in the whole body and venom gland transcriptomes of 10 chalcidoid wasp species. Light gray bars indicate gene copies that are expressed in whole body and venom gland, and dark gray bars indicate copies expressed only in the whole body. Multiple bars per species indicated distinct GH19 chitinase paralogs. Expression is measured in FPKM. Phylogeny represents the most likely species tree for the chalcidoid wasps, adapted from previous studies (Raychoudhury et al. 2009; Munro et al. 2011).

A

		Chalcidoid Venoms	Chalcidoids	Mosquitoes	Rozella & Mitosporidium	Microsporidia	Oomycetes	Plants	Bacteria
Chitin cleavage residues	Glu 67	100	95	86	100	96	93	87	100
	Glu 89	100	95	100	100	96	100	98	88
	Ser 120	100	90	100	100	100	87	83	100
Chitin binding residues	Gln 118	100	90	100	100	100	100	84	88
	Asn 124	100	95	100	100	100	100	99	100
	Ile 198	100	100	71	100	100	100	96	94
	Asn 199	100	95	43	100	100	100	83	100
	Glu 203	100	100	43	100	100	100	87	100
	Arg 215	100	100	43	100	100	100	88	100
Total GH19 seqs		20	7	7	3	23	15	101	17

B

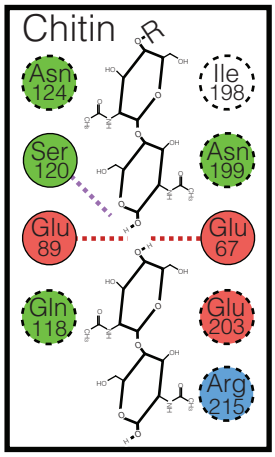

Fig. 3. GH19 catalytic residue conservation. (*A*) The percentage of GH19 chitinases that have a conserved or similar amino acid at each residue proposed to play a role in chitin cleaving or binding in other organisms. Residue positions are in reference to the *Hordeum vulgare* chitinase 2BAA, P23951. (*B*) Diagram of the catalytic mechanism of GH19 chitinases (modified from Ohnuma et al. 2014).

reservoir of *N. vitripennis* and all GH19 chitinases with high venom expression have signal peptides (supplementary fig. S1, Supplementary Material online), which together show that the gene is translated and secreted into the reservoir to be injected into hosts. Venom transcriptomes were not available for *N. oneida* or *M. raptor*; however, based on their phylogenetic positions and presence of a signal peptide it is likely that their single GH19 chitinase copy is also a component of their venom.

GH19 chitinase may additionally perform nonvenom functions. The venom copy of *N. vitripennis* GH19 chitinase was shown in genome-wide expression studies to be expressed almost equally in male and female adults and is expressed in pupae as well (Wang et al. 2013, 2015). Additionally, GH19 chitinase expression was identified in the whole body transcriptome of four species, but did not have expression in their venom glands (*Trichomalopsis sarcophagae*, *Urolepis rufipes*, *Spalangia endius*, and *S. cameroni*) (fig. 2). Further studies are needed to determine the exact nature of the nonvenom functions of GH19 chitinase in chalcidoids.

Most chalcidoid species express a single copy of GH19 chitinase in their whole body transcriptomes with the exception of *S. endius* (two copies), *S. cameroni* (two copies), *Melittobia* sp (three copies), and *Ta. zealandicus* (four copies) (fig. 1). When considering all chalcidoid wasp GH19 chitinases, gene copies do not form distinct paralog groups, nor do they recapitulate the species tree (fig. 1). In contrast, GH19 chitinases in the Pteromalidae family of wasps have a single expressed copy of GH19 chitinase that forms a monophyletic clade and recapitulates the species tree (fig. 1). This clade is also noteworthy because it contains six of the seven venom-expressed GH19 chitinase copies.

An analysis on the monophyletic clade of Pteromalidae GH19 chitinases reveals strong directional selection in some lineages (fig. 1, supplementary fig. S4, Supplementary Material online). There is significant variation in selection among branches (CodeML Likelihood ratio test $P < 0.01$). Several branches leading to clades in which GH19 chitinase has evolved into a venom, show signs of strong positive selection with a dN/dS > 1. This includes branches leading to the *Muscidifurax* clade, the *Nasonia* clade, and *N. oneida* (fig. 1, supplementary fig. S4, Supplementary Material online). These results remain significant if all chalcidoid GH19 chitinase copies are included in the tree (data not shown). Positive selection on branches leading to venom-expressed GH19 chitinases could reflect evolutionary pressures on the protein to perform new functions within the envenomated host, as these wasp species parasitize different insects.

Comparative Protein Structure of the Parasitoid GH19 Chitinase

Phylogenetic and structural predictions (see below) classify the chalcidoid LGT as a GH19 chitinase. Previously identified functional sites in these chitinases are conserved in all the parasitoid venom GH19 chitinases (fig. 3, supplementary fig. S1, Supplementary Material online). The catalytic residues responsible for chitin cleavage in this enzyme family have been characterized by site-directed mutagenesis of GH19 chitinases from plants, microsporidia, and bacteria (Ohnuma et al. 2013, 2014; Han et al. 2015). GH19 chitinases cleave chitin through an inverting mechanism, facilitated by two glutamic acids that act as the general acid and base (Glu 67 and Glu 89 numbered according to *Hordeum vulgare* chitinase 2BAA, P23951) (Brameld and Goddard 1998; Ohnuma et al. 2014) and are fully conserved in the parasitoid venom GH19 chitinases. Additionally, seven residues responsible for oligosaccharide binding during catalysis, which were identified from the crystal structure of a bacterial GH19 chitinase in complex with chitin (Ohnuma et al. 2014), were also fully conserved (fig. 3). The GH19 chitinase in wasp venom is present in the proteome, has a signal peptide, and retains the conserved catalytic sites found in functional chitinases.

Knockdown of Venom GH19 Chitinase Reveals Effect in Envenomated Hosts

To investigate possible functions of GH19 chitinase in venom, we generated GH19 chitinase-deficient venom via RNAi knockdown in *N. vitripennis* to compare to whole venom controls. This method has been shown to be highly effective in both knocking down gene expression in the venom gland and reducing or eliminating the corresponding venom protein in the reservoir (Siebert et al. 2015; Werren JH, Siebert AL, Martinson EO, He M, Edwards R, unpublished data). Venoms are produced by parasitic wasps, but their phenotypic effects are not revealed until they are injected into another organism. Wasps were allowed to sting hosts and venom effects were assessed with RNA sequencing of the host 72 h postenvenomation (for full description see Materials and Methods). The knockdown of GH19 chitinase (GH19) resulted in a reduction of average expression by 88% relative to whole venom controls in adult female wasps. There were no detectable differences in the development or behavior of the adult wasps in which GH19 chitinase was knocked down, additionally envenomated hosts showed full developmental arrest and did not display any physical differences from a whole venom sting.

GH19 chitinase knockdown venom had an extremely targeted effect on host gene expression. Only nine genes showed significantly altered expression between GH19 knockdown and whole venom control hosts (DESeq adjusted $P < 0.05$). When compared with a previous study of gene expression differences between envenomated and normally developing (nonenvenomated) hosts (Martinson et al. 2014), the GH19 knockdown showed expression levels very similar to nonenvenomated hosts for eight of the nine differentially expressed genes. The GH19 knockdown recapitulates an average of 93% of the change between envenomated and nonenvenomated hosts (fig. 4, supplementary fig. S5, Supplementary Material online). Therefore, GH19 chitinase appears to be the major venom component responsible for altering the expression of these eight host genes. *Nasonia vitripennis* venom contains a GH18 chitinase protein (*Cht5*) that might be functionally redundant and obscure additional effects of GH19 chitinase (De Graaf et al. 2010). Therefore differentially expressed genes in this knock down are GH19 chitinase specific. Of particular interest are two genes (Chitinase 8 and spatzle 3) that have annotations related to defense against fungi.

GH19 chitinase is predicted to be a functional chitinase and may cleave chitin upon envenomation, we identify a differentially expressed host gene also involved with chitin metabolism (chance probability of a differentially expressed chitinase gene, $P = 0.01$). Chitinase 8 (*Cht8*) had the largest change in expression when GH19 chitinase was present (23-fold increase, recapitulating 98% of the full venom effect on envenomated host) (fig. 4). *Cht8* is predicted to be involved in chitin binding and chitinase activity as a member of the GH18, which are widespread among insects (Adams et al. 2000). Specifically, *Cht8* has been proposed to play a role in immunity against gut infections in the flour beetle *Tribolium* (Zhu, Arakane, Beeman, et al. 2008) and

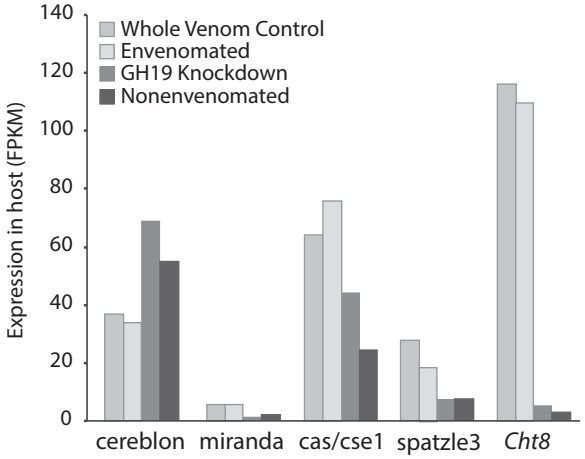

Fig. 4. Annotated genes of *Sarcophaga bullata*, the fly host, that are significantly differentially expressed upon envenomation by *N. vitripennis* with GH19 chitinase knocked down via RNAi. Comparison of host gene expression 72 h after envenomation with the complete venom repertoire "Envenomated," with *LacZ* RNAi control venom "Whole Venom Control," and venom depleted of GH19 chitinase by RNAi "GH19 Knockdown". The "Nonenvenomated" represents gene expression in normally developing hosts at the same age. Expression is measured in FPKM. The "Envenomated" and "Nonenvenomated" data are from Martinson et al. (2014).

Anopheles (Shi and Paskewitz 2004; Zhu, Arakane, Banerjee, et al. 2008), as well as proposed as a fungal-specific defense in *Apis* (Aronstein et al. 2010; Aronstein and Holloway 2013). Spatzel 3, which was also upregulated in the presence of GH19 chitinase (fig. 4), regulates expression of antifungal peptides in *Drosophila* as part of the toll pathway (Lemaitre et al. 1996; Weber et al. 2003). Although *Nasonia* venom is known to depress host immune responses to allow wasp larvae to feed undeterred (Rivers, Ruggiero, et al. 2002), previous studies have found several antimicrobial pathways are upregulated in envenomated hosts potentially preventing infections by opportunistic microbes (Danneels et al. 2013; Martinson et al. 2014).

GH19 proteins are utilized as antifungal defense in plants; therefore the chalcidoid venom GH19 chitinase may similarly, upon envenomation, defend the host from fungal infection. Our RNAi knockdown results indicate that the *N. vitripennis* venom GH19 chitinase could potentially be priming other antifungal immune responses in the host to preemptively ward off fungal infections that could lower the nutritional quality of the host. The upregulation of *Cht8* and spatzle 3 could potentially be triggered by GH19 chitinase cleaving host chitin (or fungal chitin already present in the host) that could be recognized by the host as a foreign invasion. Alternatively, the venom GH19 chitinase could be injuring the host by breaking down host chitin structures, allowing easier access to host nutritional contents for the wasp offspring. However, the final breakdown products of chitin degradation do not increase in envenomated hosts (Mrinalini et al. 2014), and therefore the antifungal hypothesis is currently favored.

The remaining differentially expressed genes are annotated as miranda, cas/cse1 segregation protein, and cereblon (fig. 4).

Four genes did not have annotations or structural predictions (supplementary fig. S5, Supplementary Material online). Cereblon was the only gene downregulated in the presence of GH19 chitinase, which is an ubiquitin ligase regulator involved in developmental processes (Epling-Burnette et al. 2013). Genes upregulated due to GH19 chitinase include two that are involved in the transport of proteins that provide developmental cues (annotated as cas/cse1 segregation protein and miranda) (Tekotte et al. 2002; Yousef et al. 2008).

Genomic Context and Gene Structure of Chalcidoid GH19 Chitinases

GH19 chitinase copy number varies among the three species with sequenced genomes (*T. pretiosum*: 4, *Co. floridanum*: 3, *N. vitripennis*: 2), and each of the GH19 chitinase copies is on a large scaffold (average 3.4 Mb) that does not contain other microsporidial or microbial genes (supplementary fig. S2 and table S1, Supplementary Material online). The wasp genes immediately flanking GH19 chitinases do not share synteny across species, which is not unexpected as the three species are from distantly related families (supplementary fig. S2, Supplementary Material online).

The gene structures of parasitoid GH19 chitinases have features consistent with an ancient LGT followed by incorporation into a metazoan genome. Although microsporidia have a single GH19 chitinase with no introns, all three chalcidoids (*N. vitripennis*, *Co. floridanum*, *T. pretiosum*) have a pair of tandem GH19 chitinase genes with introns (supplementary fig. S3, Supplementary Material online). Only one of the two copies found in the *N. vitripennis* genome is expressed (Wang et al. 2013). Upon closer inspection the nonexpressed copy displays signs of degradation (several stop codons throughout the exons) and is most likely a pseudogene (supplementary fig. S3, Supplementary Material online). In all three species, one of the tandem pair always contains an intron at amino acid site 80 (relative to the *N. vitripennis* Nasvi2EG021382 sequence). The other copy of the tandem set harbors an intron at site 158 in *T. pretiosum*, but at site 60 in *Co. floridanum* and *N. vitripennis*, suggesting that these particular introns were formed after the divergence of *T. pretiosum* and *Co. floridanum* (supplementary fig. S3, Supplementary Material online). The shared duplicate genes and homologous intron sites suggest that GH19 chitinase was incorporated into chalcidoid wasps and duplicated shortly after the transfer from microsporidia/*Rozella*. One copy then acquired an intron relatively early in its evolution.

An Independent LGT Of GH19 Chitinase from Microsporidia into Mosquitoes

Our analysis reveals a second GH19 chitinase LGT in insects. GH19 chitinase genes are present in the Culicinae mosquitoes *Culex quinquefasciatus* and *Aedes aegypti*, but are absent in their sister lineage (22 *Anopheles* sp. genomes) and all other Diptera (64 genomes) (supplementary fig. S6, Supplementary Material online). GH19 chitinase additionally occurs in the transcriptomes of *Aedes albopictus* (Poelchau et al. 2013) and *Culex tarsalis* (Calvo et al. 2010), but low sequence quality prevented their inclusion in our analyses. There is a single copy of the gene with no introns in *A. aegypti*, whereas *C. quinquefasciatus* has four copies of the gene that all contain introns (supplementary fig. S3, Supplementary Material online). For a full description of introns in the mosquito gene copies, see supplementary results., Supplementary Material online.

The phylogenetic tree shows full posterior probability and bootstrap support for a clade containing microsporidia, two insect groups (chalcidoid wasps and some mosquitoes), and *Rozella* (fig. 1). The mosquito clade is nested within the microsporidia/*Rozella* clade but separate from the chalcidoid wasp clade, indicating a second LGT from microsporidia into insects (fig. 1). Furthermore, Hymenoptera and Diptera are separated by an estimated 345 My (Misof et al. 2014) and no other holometabolous insect genome contains a GH19 chitinase (supplementary fig. S7, Supplementary Material online). A single LGT to the common ancestor of chalcidoid wasps and mosquitoes would require a minimum of nine independent losses of the gene during insect evolution (supplementary fig. S4, Supplementary Material online). We therefore conclude that the most parsimonious interpretation based on phylogenetic reconstruction is that there were two independent lateral transfers of GH19 chitinase: 1) microsporidia/*Rozella* to chalcidoid wasps and 2) microsporidia to Culicinae mosquitoes (fig. 1B). The chalcidoid GH19 chitinase was acquired at least 95 Ma near the origin of Chalcidoidea (Munro et al. 2011), whereas the transfer to mosquitoes was likely less than 39 Ma, after *Culex* and *Aedes* diverged from *Anopheles* (Foley et al. 1998).

Microsporidia are common intracellular pathogens of insects (Becnel and Andreadis 1999), which would allow for the intimate intergenomic interactions necessary for these LGTs to occur. The GH19 chitinase function in mosquitoes is currently unknown, but RNA-Seq data from previous studies show that the GH19 chitinase in *A. aegypti* (EAT34499) is upregulated in 12–24 h old embryos, testes, and ovaries after blood meal (Akbari et al. 2013). We point out that the presence of the GH19 chitinase gene in disease vectoring insects could make GH19 chitinase knockdown a tool for targeted vector control, particularly if it provides protection from pathogens in the mosquito.

Evolutionary Paths for Multiple LGTs of GH19 Chitinase through the Tree of Life

Further tracking of the GH19 chitinase gene outside of the Metazoa reveals a patchy distribution across the tree of life, indicative of multiple LGTs. In addition to chalcidoids and mosquitoes, GH19 chitinase is harbored in nearly every member of the distantly related clades microsporidia–cryptomycota, plants, and oomycetes, yet each of their sister lineages and surrounding taxa lack this gene (fig. 1B, supplementary fig. S7, Supplementary Material online). Outside of eukaryotes, GH19 chitinases occur in bacteria where it also has a sporadic distribution. (supplementary

fig. S8, Supplementary Material online). Pairwise protein structure alignments revealed remarkable structural similarity among GH19 chitinases from these disparate groups (root-mean-square deviations [RMSD] of 1.5–2.2) and all contain a GH19 domain (PF00182.15) (supplementary fig. S9, Supplementary Material online). Hence, despite the unusual, patchy phylogenetic distribution, protein sequence, and structure similarity suggest that GH19 chitinases share a common origin and have largely retained chitin-degrading functional sites (supplementary fig. S1, Supplementary Material online).

Although the microsporidia/*Rozella* origin of chalcidoid and mosquito GH19 chitinases is well supported, the donor-recipient relationships between the plants, bacteria, and oomycetes clades are more uncertain (fig. 1). Each forms a clearly separated clade, but the typical pattern observed in recent LGTs, with the receiver nested among potential donors, is not apparent. Instead the GH19 chitinase tree indicates ancient LGTs occurring at or near the base of each recipient clade (supplementary fig. S4, Supplementary Material online). These relationships may have been obscured by the rapid sequence evolution known to occur in bacteria, oomycetes, and microsporidia (Keeling and Fast 2002; Beakes et al. 2012), the high incidence of LGTs among prokaryotes, and the different forces acting on genome evolution among prokaryotes and eukaryotes (e.g., codon bias, intron formation/removal, etc.).

All microsporidia (25/25) and *Rozella* (1/1) have at least one copy of GH19 chitinase, whereas this gene is absent from all other fungal genomes (532 genomes) (Campbell et al. 2013; James et al. 2013) (supplementary fig. S7, Supplementary Material online). In the GH19 chitinase phylogeny, oomycetes are sister to the microsporidia/*Rozella* clade, and may have been the source of lateral transfer to a common ancestor of *Rozella* and microsporidia (fig. 1). A single copy of GH19 chitinase is present in most oomycete taxa (24/30) and it is predicted to be a member of the ancestral suite of secreted proteins, which digest external resources (Misner et al. 2015). We cannot infer the exact phylogenetic point that GH19 chitinase was transferred, due to a lack of genomes for early-branching oomycete lineages. However, GH19 chitinase is absent in the sister species of oomycetes (*Hyphochytrium catenoides*) (Misner et al. 2015), as well as in the numerous available genomes of related taxa (supplementary fig. S7, Supplementary Material online). Phylogenetic reconstruction does not provide definitive support, but the likely point of origin for the GH19 chitinase gene appears to be bacteria or plants. For a full discussion of GH19 chitinase in bacteria and plants and hypotheses on its origin, see supplementary results (Supplementary Material online).

Potential ecological links between taxa may have facilitated multiple LGTs across the tree of life. As previously mentioned, microsporidia are intracellular pathogens of insects (Becnel and Andreadis 1999). Additionally, Cryptomycota (*Rozella*) are common parasites of oomycetes (Gleason et al. 2014). Oomycetes in-turn are pathogens of many organisms (including plants), and their ancestral lifestyle is thought to be saprotrophic in freshwater or soil (Beakes et al. 2012), which facilitates both bacterial and plant interactions. Microsporidia genomes contain many LGTs including some that are consistent with a host-to-parasite transfer event (i.e., *Daphnia*, insects), which have been hypothesized to aid in exploitation of the host (Selman and Corradi 2011; Pombert et al. 2012, 2015; Parisot et al. 2014). The GH19 chitinases in Chalcidoidea wasps and Culicinae mosquitoes may be examples of the mirror parasite-to-host LGT, which may lead to a novel function in the host not associated with defense against the parasite.

Conclusion

GH19 chitinase is an unusual LGT in that 1) it has been laterally transferred independently multiple times and 2) it is the first insect LGT from a eukaryotic donor that does not involve carotenoid synthesis genes. Sequence homology, protein structure, and phylogenetic relationships tie the evolutionary history of the GH19 chitinase lineages together. Organisms harboring this gene use it for diverse functions, ranging from nutrient acquisition in bacteria and oomycetes (Davies and Henrissat 1995; Bhattacharya et al. 2007), defense in plants (Fritig et al. 1998; Van Loon et al. 2006), and structural reorganization in microsporidia (Roennebaeumer et al. 2006). In this study, we show that several species of chalcidoid wasps use GH19 chitinase as a venom that significantly changes the expression of genes in envenomated hosts involved in fungal protection. The origin of genetic novelty is a major question in science and this study provides evidence for chalcidoid wasps having acquired a gene through lateral transfer, and subsequently incorporating the novel function into its venom repertoire.

Materials and Methods

Genome and Database Searches

The *N. vitripennis* GH19 chitinase was used to find orthologs in OrthoDB (v8) and to query the NCBI nr database for an initial survey of this gene family. A BLASTp cutoff of $1e^{-15}$ was used to identify orthologs in bacteria, archaea, viruses, and eukaryotes (excluding Angiosperms). The diversity of closely related GH19 chitinases is extensive within plants, therefore, a BLASTp search was performed across Viridiplantae within Phytozome v9.1 (Goodstein et al. 2012) (accessed January 2015). To further search for GH19 chitinase genes, genomes were downloaded for microsporidia, oomycetes, insects, Stramenopiles, Rhizaria, Alveolates, Cryptophytes, and Haptophytes, and were searched with tBLASTn using the same cutoffs as above to locate open reading frames that had not been previously annotated. In-depth searches in oomycetes were also performed at the Pythium Genome Database (http://pythium.plantbiology.msu.edu) (accessed January 2015) (supplementary fig. S7 and S8, Supplementary Material online).

Genomic Location and Introns

The presence and placement of introns and flanking genes were identified using BLASTp in the four currently sequenced

chalcidoid genomes: *N. vitripennis*, *C. solmsi*, *T. pretiosum*, and *Co. floridanum* (i5K_Consortium 2013), as well as sequenced microsporidia genomes and the mosquitoes *Culex* quinquefasciatus and *Aedes aegypti*.

Transcriptome Searches

Homologous GH19 chitinase genes were identified by BLASTp searches in the transcriptomes of whole body adult female wasps of 13 chalcidoid species (*N. vitripennis*, *N. giraulti*, *N. oneida*, *N. longicornis*, *Trichomalopsis sarcophagae*, *U. rufipes*, *M. uniraptor*, *M. raptor*, *M. raptorellus*, *S. endius*, *S. cameroni*, *Melittobia* sp, and *Ta. zealandicus*). Each transcriptome was a pool of six individuals. To assess gene expression in the venom gland, BLASTp searches were also conducted on venom transcriptomes from 10 species of chalcidoid wasp (*N. vitripennis*, *N. giraulti*, *N. longicornis*, *Trichomalopsis sarcophagae*, *U. rufipes*, *M. uniraptor*, *M. raptorellus*, *S. cameroni*, *Melittobia* sp, and *Ta. zealandicus*). The venom gland transcriptomes contained 50 pooled whole venom apparatuses, including venom gland and reservoir. Full details on transcriptome assembly can be found in the supplementary materials and methods, Supplementary Material online.

Phylogenetic Analyses

Protein sequences of the GH19 chitinase gene were aligned with MAFFT (Katoh et al. 2002) using the default settings. The final alignment was trimmed at the boundaries of the GH19 chitinase domain to avoid comparing nonconserved regions present in only a subset of the taxa. ProtTest3.4 (Darriba et al. 2011) was used to identify the best-fit model of amino acid substitution for the alignment using the Bayesian information criterion (BIC) and Akaike information criterion (AIC) scores. Phylogenetic reconstructions were performed with maximum-likelihood and Bayesian inference using RAxML 8.0.0 (Stamatakis 2006) and MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003), respectively. The maximum-likelihood tree was run for 1,000 bootstrap replicates and the Bayesian tree was run for 10,000,000 generations with a burn-in of 25%. The final tree included sequences representative of the embryophyte diversity, bacteria, oomycetes, insects, and microsporidia.

CodeML from the PAML software package (Yang 2007) was used to test for positive selection for particular nodes by estimating omega, the ratio of nonsynonymous changes per nonsynonymous site to synonymous changes per synonymous site (dN/dS). The unconstrained free-ratio model (independent omega values for every branch) was used to estimate variation in omega across the phylogeny compared the one-ratio model (omega is fixed across all branches). Each model was run on multiple trees including various numbers of species and GH19 chitinase paralogs to test if the pattern was robust. Additionally, to compare orthologous sequences, we tested a subset of GH19 chitinases that form a monophyletic clade of single copy genes with the addition of the single *Ta. zealandicus* venom copy and a microsporidial outgroup (supplementary fig. S4, Supplementary Material online).

RNAi Knockdown of the *N. vitripennis* GH19 Chitinase

To examine possible functions of GH19 chitinase venom, *N. vitripennis* larvae were injected with dsRNA designed from the *N. vitripennis* venom GH19 chitinase gene sequence (Nasvi2EG021382) or with dsRNA LacZ control as per the protocol described by (Werren et al. 2009) that has been shown to successfully knockdown venom proteins (Siebert et al. 2015). Larvae were reared to adulthood, resulting in wasps with depleted expression of a single venom component (GH19 chitinase "GH19" knockdown), or wasps with the complete venom set (whole venom controls). Females from both groups were presented with *Sarcophaga bullata* hosts for 8 h to allow feeding and practice stinging, after which they were removed and starved overnight. The following morning, females were set individually on *Sa. bullata* pupae placed in drilled foam plugs to ensure exposure of only the anterior end of the host puparium for stinging and oviposition. The wasps were allowed 4 h to envenomate hosts, then placed directly into TRIzol Reagent (Ambion) in groups of five and stored at −80 °C for subsequent confirmation of GH19 knockdown by semiquantitative polymerase chain reaction (PCR). Wasp RNA was extracted per manufacturer's protocol (Ambion) and concentrations were determined using a NanoDrop 1000 Spectrophotometer. First strand cDNA was made using RevertAid H Minus First Strand cDNA Synthesis K (Thermo Scientific). Semiquantitative PCR was performed per manufacturer protocol using 10 ul reactions with GoTaq Green (Promega) and primers endoCH_F3 GATGCG TACGTTTATTCTCG and endoCH_R3 AACCTTGAGGTAGA TCTGGT. Cycle conditions were 95 °C for 2 min, 95 °C for 30 s, 58 °C for 45 s, 72 °C for 1 min, for 17, 20, 25, and 30 cycles, final annealing of 5 min at 72 °C, and a hold at 4 °C. All samples were run on a 2% agarose Et-Br gel and band luminescence was quantified using ImageJ (NIH). Knockdown efficiency was estimated by comparing the luminescence ratio of the GH19 chitinase gene to a control gene, ribosomal protein 49, and the resulting ratios were compared between GH19 knockdown and whole venom control wasps.

After the wasps were removed, all *Sa. bullata* hosts had their anterior pupal cap removed to verify a sting site and remove any eggs laid by *N. vitripennis*. Hosts were stored at 25 °C and allowed to develop with a gel capsule (Medisa Inc.) covering their exposed anterior end to prevent dehydration. Hosts were sampled 72 h after envenomation and stored in TRIzol Reagent (Ambion) at −80 °C. Extraction was performed as per manufacturer's protocol (Ambion), and RNA quality and concentration were determined using an Agilent 2100 bioanalyzer. Three pools of five whole venom control hosts and three individual GH19 knockdown hosts were used for six TruSeq mRNA (Illumina) library constructions and sequencing on an Illumina HiSeq2500 Sequencer at the URGRC. Each library was sequenced on 1/12th of one lane.

Processing of the raw Illumina reads was completed as described in supplementary materials and methods, Supplementary Material online. Cleaned reads for each condition and replicate were mapped to a previously published

Sa. bullata transcriptome (Martinson et al. 2014) using Tophat2 v2.0.4 (default settings) for FPKM values (Trapnell et al. 2012, 2013). Count data were generated using HTSeq-count with the intersection-strict mode (Kim et al. 2013). DESeq v1.12.1 was used to generate normalized read counts and differential expression calls for each treatment (adjusted P value < 0.05) (Anders and Huber 2010).

Protein Structure Predictions and Alignments

The online server Raptor X (Källberg et al. 2012) was used to predict protein structures of representative sequences from across the GH19 chitinase taxonomic diversity and to identify the best protein models. These structures were then aligned pairwise with the Raptor X protein alignment server and RMSD was used to assess alignment quality.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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