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Recurrent DNA virus domestication leading to different parasite virulence strategies

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Relics of ancient infections are abundant in eukaryote genomes, but little is known about how they evolve when they confer a functional benefit on their host. We show here, for the first time, that the virus-like particles shown to protect Venturia canescens eggs against host immunity are derived from a nudivirus genome incorporated by the parasitic wasp into its own genetic material. Nudivirus hijacking was also at the origin of protective particles from braconid wasps. However, we show here that the viral genes produce “liposomes” that wrap and deliver V. canescens virulence proteins, whereas the particles are used as gene transfer agents in braconid wasps. Our findings indicate that virus domestication has occurred repeatedly during parasitic wasp evolution but with different evolutionary trajectories after endogenization, resulting in different virulence molecule delivery strategies.

INTRODUCTION

Virus endogenization appears to be a frequent, ongoing process in the evolution of eukaryotic genomes (1, 2). Polydnaviruses constitute a unique example in which eukaryotes maintain a complex viral machinery in their genomes, providing a function essential for their life cycle. Polydnaviruses are carried by thousands of species of parasitic wasps that develop within living insect larvae (3). The wasp makes use of these viruses to establish successful parasitism in hosts with competent immune defenses. Polydnaviruses include the bracoviruses and ichnoviruses associated with braconid and ichneumonid wasps, respectively. Both groups of viruses mediate the transfer of virulence genes (4) and have evolved by convergence, through two genome integration events involving the insertion of DNA from unrelated viruses into wasp chromosomes (5, 6). However, an enigma remains regarding the nature of the first particles from parasitic wasps to be described, produced in a species from the group of ichnovirus-carrying wasps, Venturia canescens. This wasp uses “virus-like particles” (VLPs) to protect its eggs against the host immune response (7–10). VLPs are produced in the ovaries and released into the ovary lumen, where they become associated with the eggs. In the parasitized host, they protect the eggs against encapsulation, an insect immune response involving the engulfment of foreign bodies in a sheath of immune cells. Unlike polydnaviruses, which convey virulence genes, VLPs are devoid of DNA (10, 11) and enclose virulence proteins (12). VLPs do not resemble the particles of known viruses, and a debate about whether these particles are truly of viral origin has raged since the early 1970s (12). We show here that VLPs are clearly of viral origin and consist of virulence proteins wrapped in viral envelopes. They are produced by a machinery derived from the domestication of a nudivirus [a sister group of baculoviruses (13)]. This domestication occurred through an event independent of the capture of a nudivirus at the origin of braconviruses (14). Thus, similar viruses have been acquired in different wasp lineages but have followed different evolutionary trajectories after endogenization.

RESULTS

A nudiviral machinery produces V. canescens VLPs

We first investigated the nature of the VLPs using a comprehensive deep sequencing analysis of the mRNAs synthesized in the tissues producing them. An analysis of the calyx transcriptome led to the identification of 51 abundant transcripts displaying significant similarities to nudivirus genes (Table 1 and table S1). Polymerase chain reaction (PCR) amplification with DNA from various sources showed that all of the wasp specimens analyzed contained nudiviral genes (fig. S1A), which were therefore probably fixed in V. canescens and did not correspond to a pathogenic virus infecting the laboratory strain.

The hypothesis on a role for these nudiviral genes in VLP production was further supported by the nature of the transcripts. Indeed, nudiviruses have 21 conserved genes involved in essential viral functions in common with baculoviruses (15), and transcripts corresponding to 16 of these genes were identified in V. canescens ovaries. Their function in the baculovirus life cycle has been characterized [reviewed by Rohrmann (16)], and we can infer that nudiviral homologs may have a similar role. The VLP-producing tissue expressed homologs of the seven genes encoding viral envelope proteins [named per os infectivity factors (PIFs)] mediating the entry of particles into target cells. We also detected the expression of four genes (lef-4, lef-8, lef-9, and p47) encoding the subunits of the viral RNA polymerase controlling the transcription of a series of viral genes. Conversely, no viral DNA polymerase or capsid protein (for example, VP39, 38K, and VLF-1) transcripts were detected, consistent with the absence of nucleocapsids.
(DNA packaged in structural proteins) in the VLPs. A role for nudivirus genes in VLP production was also supported by their temporal and tissue-specific patterns of expression, as assessed by quantitative reverse transcription PCR (RT-PCR), which were strongly correlated with VLP production (fig. S1). Finally, transmission electron microscopy (TEM) studies of VLP production in wasp ovary cells showed that the morphogenesis of VLPs resembled that of nudiviruses (17) and bracoviruses (18). Indeed, VLPs form in the nucleus (Fig. 1A) from a "virogenic stroma" (the "viral factory" in which virus particles are generally assembled) and a network of viral envelopes (Fig. 1, B and C) typical of these viruses. This results in the production of enveloped particles, which leave the nucleus through the nuclear envelope and are released from the cell by budding from the plasma membrane (Fig. 1D).

We checked whether the nudiviral genes produced VLPs by analyzing proteins from purified particles using liquid chromatography tandem mass spectrometry (LC-MS/MS). We identified 26 nudiviral gene products, including the whole set of PIF envelope proteins, as components of the

Table 1. Nudiviral and virulence genes expressed in V. canescens ovaries and the presence of their products in VLPs. Nudiviral genes of unknown function are detailed in table S1. RPKM, number of Illumina reads per kilobase per million mapped reads. PSM, number of peptide spectrum matches.

| Protein putative function | Gene name | Transcript abundance (RPKM) | Peptide abundance (PSM) | OrNV homolog | Accession number | BLASTp e value |
|---------------------------|-----------|-----------------------------|------------------------|---------------|-----------------|----------------|
| Nudiviral genes encoding VLP components | | | | | |
| Envelope component | pif-0 (p74) | 12,900 | 54 | P74 protein (OrNV_gp126) | YP_002321437 | 1.0 × 10^{−85} |
| | pif-1 | 5,196 | 28 | PIF-1 (OrNV_gp060) | YP_002321371 | 2.0 × 10^{−94} |
| | pif-2 | 1,980 | 34 | PIF-2 (OrNV_gp017) | YP_002321328 | 9.8 × 10^{−112} |
| | pif-3 | 2,655 | 12 | PIF-3 (OrNV_gp107) | YP_002321418 | 2.0 × 10^{−59} |
| | pif-4 | 7,731 | 5 | 19-kDa protein (OrNV_gp033) | YP_002321344 | 5.0 × 10^{−44} |
| | pif-5-1 | 527 | 5 | ODV-E56 (OrNV_gp115) | YP_002321426 | 1.0 × 10^{−34} |
| | pif-5-2 | 863 | 21 | ODV-E56 (OrNV_gp115) | YP_002321426 | 4.8 × 10^{−38} |
| | pif-5-3 | 1,048 | 9 | ODV-E56 (OrNV_gp115) | YP_002321426 | 1.6 × 10^{−29} |
| | pif-6 | 3,011 | 2 | Ac68-like (OrNV_gp072) | YP_002321383 | 4.0 × 10^{−40} |
| | vp91 | 2,966 | 23 | VP91 (OrNV_gp106) | YP_002321417 | 1.4 × 10^{−84} |
| | p33 | 5,553 | 5 | Ac92-like (OrNV_gp113) | YP_002321424 | 8.6 × 10^{−48} |
| Unknown | 15 other genes related to OrNV |

V. canescens genes encoding VLP components

| Virulence | PHGPx VLP1 | 1,115 | 134 |
|-----------|------------|-------|------|
| RhoGAP VLP2 | 23,134 | 400 |
| Neprylisin VLP3 | 3,540 | 138 |

Nudiviral genes transcribed in VLP-producing tissue not encoding VLP components

| Replication | Helicase | 27 | DNAHEL (OrNV_gp034) | YP_002321345 | 0.0 |
|--------------|----------|---|----------------------|-----------------|-----|
| Transcription | RNA polymerase lef-4 | 488 | — | LEF-4 (OrNV_gp042) | YP_002321353 | 2.8 × 10^{−92} |
| | RNA polymerase lef-8 | 125 | — | LEF-8 (OrNV_gp064) | YP_002321375 | 0.0 |
| | RNA polymerase lef-9 | 724 | — | LEF-9 (OrNV_gp096) | YP_002321407 | 0.0 |
| | RNA polymerase p47 | 916 | — | P47 (OrNV_gp020) | YP_002321331 | 1.6 × 10^{−97} |
| | Initiation factor lef-5 | 245 | — | LEF-5 (OrNV_gp052) | YP_002321363 | 5.5 × 10^{−18} |
| Envelope component | pif-5-4 | 2,286 | — | ODV-E56 (OrNV_gp115) | YP_002321426 | 9.1 × 10^{−22} |
| | Ac81-1 | 272 | — | Ac81-like protein (OrNV_gp004) | YP_002321315 | 2.0 × 10^{−34} |
| | Ac81-2 | 1,777 | — | Ac81-like protein (OrNV_gp004) | YP_002321315 | 1.0 × 10^{−39} |
| | Ac81-3 | 1,080 | — | Ac81-like protein (OrNV_gp004) | YP_002321315 | 9.3 × 10^{−34} |
| Unknown | 18 other genes related to OrNV |
Fig. 1. VLP morphogenesis in *V. canescens* calyx cells. (A to E) Transmission electron micrographs of (A) calyx cells displaying hypertrophic nuclei (N) containing virogenic stroma (VS) and VLPs; (B) calyx cell nuclei with empty envelopes close to the VS; (C) envelopes filled with VS material giving rise to VLPs; (D) VLPs budding from the calyx cell membrane; (E) immunogold labeling of VS and VLPs in the nucleus with specific antibodies against VLP2 (left) and PIF-0 (right). (Insets) Larger magnifications of VLPs. CYT, cytoplasm; Mv, microvilli of the cytoplasmic membrane; OvL, oviduct lumen; Pm, plasma membrane.
VLPs (Table 1). We also identified the virulence proteins of wasp origin thought to ensure immune protection: a peroxidase (VLP1), a Rho GTPase (guanosine triphosphatase)–activating protein (VLP2), and a metalloprotease (VLP3) [19–21] (Table 1)—the most abundant components of VLPs (Table 1). The presence of VLP2 and a nudiviral PIF protein in the VLPs was also confirmed by their direct visualization in immunogold labeling experiments (Fig. 1E). We conclude that VLPs consist of wasp virulence proteins wrapped in nudiviral envelopes and that they are produced by a nudiviral machinery that has lost the ability to package DNA. VLPs may thus be considered as naturally produced viral liposomes.

**Recurrent domestications of nudiviruses in parasitic wasps evolve differently**

We investigated whether the nudiviral genes were present in the wasp genome evolutionarily, and whether pathogenic nudiviruses and are not widely distributed throughout the wasp lineage. V. canescens wasp virulence proteins wrapped in nudiviral envelopes and that they formed an integral part of the genetic material of the wasp. Phylogenetic analyses indicated that nudiviral genes from the different clusters all belonged to the clade of *Oryctes rhinoceros* nudivirus (OrNV) (Fig. 2B). This suggests that VLPs are derived from the chromosomal integration of a nudivirus related to OrNV, the genome of which has undergone rearrangements leading to fragmentation and gene loss with respect to pathogenic nudiviruses (15).

The polydnaviruses associated with braconid wasps (bracoviruses) are also derived from the acquisition of a nudivirus genome (5). However, phylogenetic analyses indicated that the nudiviral genes giving rise to VLPs and bracoviruses came from different viral clades, the Alphanudiviruses and the Betanudiviruses, respectively (Fig. 2B). Moreover, V. canescens nudiviral genes are more closely related to those of pathogenic nudiviruses and are not widely distributed throughout the wasp genome, unlike most of the genes involved in bracovirus particle production (5, 22). This is consistent with a more recent acquisition in regions containing wasp genes (Fig. 2A and table S1), confirming that they formed an integral part of the genetic material of the wasp.

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**Fig. 2.** Organization, origin, and putative function of the nudiviral genes of the *V. canescens* genome. (A) Nudiviral genes (in red; named after their OrNV homologs) are grouped into six clusters flanked by regions containing conserved wasp genes (in blue). (B) Phylogenetic tree of insect large double-stranded DNA viruses obtained by maximum likelihood analysis with a concatenated multiple alignment for 37 genes showing that the nudiviruses captured by the *V. canescens* and braconid wasp lineages belonged to different clades, VcVLP, *V. canescens* VLP. (C) Genomes common to nudiviruses and baculoviruses conserved in *V. canescens* (red) or wasps carrying bracoviruses (BV) (purple). The products of the *helicase* gene and of the genes mediating viral gene transcription are not incorporated into the particles (hatched motif). Note that *V. canescens* lacks capsid genes, consistent with the absence of DNA in the particles.
**Fig. 3. Overview of the production, function, and origin of V. canescens VLPs.** (A) VLPs consist of virulence proteins of wasp origin wrapped in nudiviral envelopes. VLPs are released into the oviduct lumen, where they become attached to the wasp egg. Once in the parasitized caterpillar host, VLPs confer immune protection to the egg by impairing capsule formation by the host immune cells. (B) Recurrent virus domestication during parasitic wasp evolution. Two virus genome integration events have occurred during the evolution of campoplegine wasps: the first involved a genome, making it possible to produce ichnoviruses (mediating gene transfer), and the second involved an alphanudivirus genome used for VLP production (mediating the delivery of virulence proteins), which has replaced the ichnovirus in V. canescens. A betanudivirus was independently acquired by a braconid wasp ancestor and gave rise to bracoviruses (DNA delivery).
V. canescens than in braconid wasps. Indeed, VLPs have been described only in the species V. canescens, suggesting that alphanudivirus genome integration occurred, at most, a few million years ago, whereas betanudivirus genome integration occurred ~100 million years ago (14). Overall, our data indicate that the hijacking of nudiviruses has contributed at least twice to virulence strategies in parasitic wasps. However, the integrated nudivirus genomes have evolved differently. In particular, the structural components of the nucleocapsid, encoded by vlf-1, 38K, and vp39, have been specifically lost from V. canescens, despite the relatively recent nature of the alphanudivirus integration (Fig. 2C). This study thus provides the first documented example of related endogenous viruses evolving differently within eukaryote host genomes according to the function conferred on the host (gene transfer versus protein delivery). This is of particular interest because these viruses initially had very similar genetic potentials. Their different uses by the parasitic wasps suggest that stochastic phenomena may have played a role in endogenous virus evolution. Indeed, we hypothesize that, shortly after the initial integration of the nudivirus ancestors into the genome, regulatory sequences allowing the packaging of viral DNA into nucleocapsids may have been lost in the case of V. canescens and translocated in the case of braconid wasps, resulting in the absence of viral DNA in the particles, or its replacement by wasp DNA, conferring an advantage during parasitism.

Another unresolved question concerns the fate of the former endogenous symbiotic virus in V. canescens, which belongs to a group of parasitic wasps (23) (fig. S2A) normally associated with a vertically transmitted ichnovirus with a specific set of genes (6). We found several remnants of the endogenous ichnovirus (fig. S2B and table S2). However, the few typical ichnovirus virulence genes identified were not expressed in the calyx. Thus, in V. canescens, domestication of the nudiviral machinery has led to the replacement of ichnovirus particles with VLPs to ensure progeny survival (Fig. 3). This constitutes the first reported case of endogenous mutualistic virus replacement.

CONCLUSION
Overall, our data indicate that virus integration has occurred repeatedly during the evolution of parasitic wasps, conferring different functions (gene or protein delivery). The domestication of a whole set of virus genes conferring a new function may thus be a widespread but currently underestimated evolutionary mechanism. Thus, endogenous viruses from parasitic wasps are fascinating from an evolutionary perspective. In addition, VLPs can be considered as natural viral “liposomes” transporting virulence proteins into host target cells. They contain the whole set of envelope proteins responsible for the entry of baculovirus (16) particles into insect cells. They may, therefore, enter host hemocytes (that adhere to the wasp egg during the encapsulation process) in a similar manner, thereby protecting the egg by transferring proteins suppressing the immune response in these cells. Recent decades have seen the investment of considerable amounts of energy and money into the development of liposomes or Virus-Like-Particles for delivering molecules of therapeutic interest to target cells. We show here that the natural production of such vectors was selected in parasitic wasp lineages long before the first primates evolved and their descendants were able to develop biotechnologies. The study of the natural production of such vectors in parasitic wasps may inspire the design of new vectors for therapeutic purposes.

MATERIALS AND METHODS

Insects
The thelytokous and arrhenotokous laboratory strains of the solitary parasitoid wasp V. canescens (Ichneumonidae, Campopleginae) studied here originated from natural populations collected in the Valence area (France). The two strains were mass-reared separately on larvae of a natural host, Ephesia kuehniella (Pyralidae), maintained on maize flour in a growth chamber at 25°C ± 2°C under a 16-hour light/8-hour dark photoperiod. Wasps from Tokyo (Japan) and France (two populations from the Nice region and three populations from the Valence region) were preserved in ethanol and sent to our laboratory.

Transmission electron microscopy
Samples were treated as previously described by Doremus et al. (24). Ovaries from thelytokous wasp pupae (13-day-old pupae) were dissected in 1× phosphate-buffered saline (PBS) solution, transferred, and fixed by overnight incubation at 4°C with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and then postfixed by incubation with 2% (v/v) osmium tetroxide in the same buffer for 1 hour at room temperature. Samples were dehydrated by passage through a series of concentrations of ethanol and embedded in Epon. Ultrathin sections of calyx, contrast-stained with uranyl acetate and lead citrate, were examined under a Hitachi H7100 transmission electron microscope.

TEM immunogold labeling
Ovaries from 12-hour-old females were dissected and fixed by incubation for 1 hour in 1× PBS–4% paraformaldehyde at 4°C, washed with PBS, and dehydrated by passage through a series of ethanol solutions (30 to 100%) before progressive embedding in 100% Unicryl resin (EMS Sciences). Ultrathin sections were cut with a Diatome knife on an LKB ultramicrotome and collected on nickel grids. Immunogold electron microscopy was performed with polyclonal rabbit antibodies against the VLP2 (the wasp GTPase activating protein) or PIF-0 proteins. Sections were treated for 10 min with 50 mM NH4Cl and blocked by two incubations (for 10 min each) with 1% bovine serum albumin (BSA). The sections were washed in 1× PBS and incubated with the primary antibody diluted 1:500 in 0.5% BSA in PBS for 2 hours at room temperature. The preimmune serum was used as control. After several washes in 1× PBS, sections were incubated with goat antirabbit immunoglobulin G antibodies conjugated to 20-nm gold particles (Abcam) and diluted 1:50 in 1× PBS supplemented with 0.5% BSA for 45 min at room temperature. Finally, sections were stained with 1% acetate uranyl and examined under a Hitachi H7100 electron microscope at 80 kV.

Sequencing and assembly of the calyx transcriptome
A complementary DNA (cDNA) library was generated from total RNA extracted from the calyx of thirty 24-hour-old females from the thelytokous laboratory strain. We used the RNeasy Mini Kit (Qiagen), according to the manufacturer’s protocol, to extract RNA immediately after calyx dissection in 1× PBS. The RNA was treated with the TURBO DNA-free kit (Life Technologies) according to the manufacturer’s instructions, and the mRNA (2.17 μg) was converted into cDNA with the TruSeq RNA Sample Preparation Kit (Illumina). Paired-end sequencing [2 × 100–base pair (bp) reads] was performed on a HiSeq 2000 sequencing system (illumina) at the Genoscope national sequencing center at a depth of 187,964,116 total reads. Paired-end reads were cleaned by a three-step procedure: (i) sequencing adapters and low-quality nucleotides (quality
and were then digested in-gel with Trypsin Gold (15 ng/ml) (Promega) and alkylated (55 mM iodoacetamide at room temperature for 30 min), three washes in 50% acetonitrile and 50 mM ammonium bicarbonate.

The peptides generated were extracted from the gel matrix in 50% acetonitrile and 5% formic acid. Peptide solutions were dehydrated in a vacuum centrifuge. Samples were analyzed online by nanoflow high-performance liquid chromatography (HPLC)–nanoelectrospray ionization with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate 3000 HPLC machine (Dionex). Samples were desalted and preconcentrated online on a Pepmap precolumn (0.3 mm × 10 mm; Dionex). Peptides were subjected to HPLC on a capillary reversed-phase C18 column (0.075 mm × 150 mm; Pepmap, Dionex), from which they were eluted with a gradient of 0 to 40% B over 60 min (A = 0.1% formic acid and 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) at a flow rate of 300 nl/min. Spectra were recorded with Xcalibur v2.0.7 software (Thermo Fisher Scientific) and were acquired using the instrument operating in the information-dependent acquisition mode over the entire HPLC gradient. The mass scanning range (m/z) was 400 to 2000, the capillary temperature was 200°C, and the standard mass spectrometric conditions included an ion spray voltage of 2.20 kV, a capillary voltage of 40 V, and a tube lens voltage of 120 V. Cycles of one full-scan mass spectrum at a resolution of 60,000, followed by five data-dependent MS/MS spectra, were repeated continually throughout the nano-LC separation. MS/MS spectra, obtained with the LTQ Orbitrap XL mass spectrometer, were recorded with a normalized collision energy of 35% (activation Q = 0.25; activation time, 30 ms) and an isolation window of 2 m/z. All MS/MS spectra were used as queries against entries from a restricted database consisting of the transcriptome assembly described above, together with sequences from VLP virulence proteins obtained from NCBI and nudiviral proteins obtained from the genome assembly with Proteome Discoverer v1.4 software (Thermo Fisher Scientific) and Mascot v2.4 (Matrix Science; http://matrixscience.com). Carbamidomethylation was set as a fixed modification, and methionine oxidation was set as a variable modification for searches. Peptide identifications were accepted on the basis of Percolator results, with a false discovery rate of 1%. We considered only proteins identified with two or more peptides. We manually validated peptides aligned with nudiviral protein sequences obtained from the genome assembly and peptides aligned with three virulence proteins retrieved from the genome assembly.

**Amplification of nudiviral sequences in V. canescens wasps of different origins**

Males and females from arrenhotokous laboratory strains of *V. canescens* collected in the Valence area (France) in 2007 (Valence 1) and 2010 (Valence 2) and from a laboratory strain collected in the Nice area (France) (Nice 1) were sent to our laboratory, together with females from a strain collected in Tokyo (Japan). Twenty *V. canescens* females from wild populations in the Valence (Valence 3) and Nice (Nice 2) areas were also used in this experiment. Total DNA was extracted with the QiaAmp kit (Qiagen). *V. canescens*–specific primers for three nudiviral genes—*Ac81-3, OrNVorf18-like*, and *pif-6*—were selected for PCR amplification (primer sequences presented in Table S3). The wasp *actin* gene was used as a positive control for amplification. A 30-cycle PCR (94°C for 60 s, 58°C for 60 s, and 72°C for 60 s) was performed with 10 pmol of each primer, 0.2 mM deoxynucleotide triphosphate (dNTP; MP Biochemicals), 1.5 mM MgCl₂, 0.5 U of Goldstar (Eurgene), and 1 µl of total DNA. PCR products (8 µl) were run on 2% agarose gels. As control, PCR was also performed with DNA extracted from *Hypotermia duminator*, a related wasp species, for which no amplicon
was obtained. In all experiments, a PCR was carried out in the absence of a DNA template, as a negative control.

Expression of nudiviral genes

The transcription of nudiviral genes was quantified in *V. canescens* thelytokous female adults and pupae on the sixth [pupal stage 1 (P1)], seventh [pupal stage 2 (P2)], eighth [pupal stage 3 (P3)], and ninth [pupal stage 4 (P4)] days of pupal development. Three biological replicates were used per developmental stage. For each biological replicate and each developmental stage, the head with thorax, calyx, and ovarioles of 12 individuals were dissected in 1× PBS. Total RNA was extracted from each sample with the RNeasy Mini Kit (Qiagen). After DNase treatment with the TURBO DNA-free kit (Life Technologies), RT was performed with the TRANSCRIPTase kit (Life Technologies) with 500 ng of oligo(dT)15 primer.

For each developmental stage, the head with thorax, calyx, and ovarioles of 12 individuals were dissected in 1× PBS. Total RNA was extracted from each sample with the RNeasy Mini Kit (Qiagen). After DNase treatment with the TURBO DNA-free kit (Life Technologies), RT was carried out using the SuperScript III Reverse Transcriptase kit (Life Technologies) with 500 ng of oligo(dT)15 primer (Promega) and 1 μl of 10 mM dNTP mix (Promega). Four *V. canescens* nudiviral genes were quantified in this experiment: OrNVorf41-like, OrNVorf59-like, pif-0 (p74), and RNA polymerase lef-8. The waps genes Ef1, actin, and GAPDH were used as reference genes (primer sequences presented in Table S3). For each gene, a standard curve was prepared with plasmid DNA. Briefly, genomic DNA was extracted from thelytokous females with the Wizard Genomic DNA Purification Kit (Promega). Each gene was amplified by PCR and inserted into the pGEM-T vector (Promega) according to the manufacturer’s protocol. All of the clones generated were used to transform competent *Escherichia coli* TOP10 cells (IBA). Plasmid DNA was extracted with the QIAprep Spin MiniPrep kit (Qiagen) and serially diluted (1e2 to 1e7 plasmid copies). LightCycler 480 SYBR Green I Master Mix (Roche) was used for the analysis of RNA expression in 384-well plates (Roche). The total volume of 10 μl in each well included 5 μl of reaction mix, 2.2 or 2.6 μl of nuclease-free water, 0.2 μl of reference gene primers or 0.4 μl of nudiviral gene primers, and 2 μl of cDNA or diluted plasmid DNA. We determined cDNA levels with a LightCycler 480 System (Roche) through heating at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C for 10 s. Each sample was evaluated in triplicate, and the results were normalized with respect to the genes Ef1, GAPDH, and actin. The absolute values presented are the means of normalized results, and error bars indicate standard deviations.

Phylogenetic analysis of insect large double-stranded DNA viruses

The tree was obtained by maximum likelihood inference analysis of a concatenated multiple alignment (performed with the ClustalOmega program) of amino acid sequences corresponding to 37 viral genes from the baculovirus, nudivirus, and baculovirus lineages previously used for a similar phylogenetic analysis (15). The hytosavirus lineage was used as an outgroup. Support for nodes is indicated by maximum likelihood nonparametric bootstrap (100 replicates). The sequences used for alignment with the nudiviral genes involved in the production of *V. canescens* VLPs were obtained from the following viruses: *Cotesia congregata* bacovirus (CcBV), *Microplitis demolitor* bacovirus (MdBV), *Chelonus inanitus* bacovirus (CiBV), *Heliolitis zeas* nudivirus 1 (HzNV), *Pneuadon monodorus* nudivirus (PmNV), *Gryllos bimaculatus* nudivirus (GbNV), *Ornv*, *Drosophila innubia* nudivirus (DiNV), *Curcigenipalpus* nucleopolyhedrovirus (CuniNPV), *Nediaion spier* nucleopolyhedrovirus (NeseNPV), *Autographa california* multiple nucleopolyhedrovirus (AcMNPV), *Lymorna dispar* multiple nucleopolyhedrovirus (LdMNPV), *Cydia pomonella* granulovirus (CpGV), Musca domestica salivary gland hytosavirus (MdSGHV), and *Glossina pallidipes* salivary gland hytosavirus (GpSHV).

Wasp phylogeny analysis

A PCR product corresponding to a fragment of the cytochrome oxidase 1 (CO1) gene was obtained from *V. canescens* DNA, extracted as described above. A MegaBlast search against the GenBank database was performed to retrieve the CO1 sequences of the ichneumonid wasps *Transoxema rostrale* (HQ107907), *Campeolites sonorense* (DQ538849), *Hyposoter exiguae* (DQ538855), *Hyposoter fugitivus* (DQ538858), and *Glypta funiferae* (HQ107353); the braconid wasp *C. congregata* (DQ538816); and the pteromalid wasp *Nasonia vitripennis* (EU746534), which was used as an outgroup. Sequences were aligned using the MUSCLE algorithm (available from http://phylogeny.lirmm.fr/), and the optimal nucleotide substitution model for each locus was chosen on the basis of the Bayesian information criterion, as implemented in jModeltest 2.1. We performed a Bayesian analysis with MrBayes 3.1.2, with the selected model GTR + 8 and the following parameters: five independent Markov chain Monte Carlo iterations, 1 million generations with tree and parameter sampling occurring every 100 generations, and N. vitripennis (as the outgroup). The first 25% of trees were discarded as burn-in, leaving 750,000 trees per run, and we determined the maximum clade credibility tree with a posterior probability limit of 0.5. The resulting tree was visualized with MEGA software.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/1/e1501150/DC1

Fig. S1. Link between nudiviral genes and *V. canescens* VLPs.

Fig. S2. Vestiges of the ichnovirus in the *V. canescens* genome.

Table S1. Nudiviral and flanking wabs sequences identified in the *V. canescens* genome scaffolds.

Table S2. Selected list of ichnovirus-like sequences identified in the *V. canescens* genome.

Table S3. List of PCR and quantitative PCR primers used to amplify nudiviral genes and wasp housekeeping genes.

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