# Genomics and reproductive biology of *Leptopilina n. sp.* Buffington, Lue, Davis & Tracey sp. nov. (Hymenoptera: Figitidae): An asexual parasitoid of Caribbean *Drosophila*

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**ABSTRACT**

*Drosophila* and parasitic wasps in the genus *Leptopilina* have long been a model for understanding host-parasite interactions. Indeed, parasitic wasps are important drivers of ecological and evolutionary processes broadly, but we are generally lacking information about the diversity, natural history, and evolution of these relationships. We collected insects from the Caribbean Island of Saint Lucia, home to the eastern Caribbean '*dunni*' subgroup of *Drosophila*: a clade long appreciated for its recent patterns of speciation and adaptation. Here we present an integrative approach that incorporates natural history, taxonomy, physiology, and genomics to describe *Leptopilina n. sp.* Buffington, Lue, Davis & Tracey sp. nov. (Hymenoptera: Figitidae), a virulent parasitoid of *dunni* group flies, especially *Drosophila antillea.* *Leptopilina n. sp.* is nested within an early-branching clade of *Leptopilina*, offering insights into the evolution of this important genus of *Drosophila* parasitoids. We present a high-quality assembly for this wasp's 1Gbp genome, and for its bacterial endosymbiont: *Wolbachia* strain "*w*Lmal". Furthermore, we show that *w*Lmal induces parthenogenesis in the wasp, and that these wasps are reliant upon their *Wolbachia* infections to produce female offspring. Finally, comparison to historical museum specimens indicate that *Leptopilina n. sp.* had been collected approximately 40 years prior from the nearby island of Guadeloupe and were also asexually reproducing. This work represents one of only a handful of studies in which field biology, taxonomy, systematics, genomics, and experimental biology are integrated into a species description: showcasing the possibilities for biodiversity research in the genomic era.

**KEYWORDS**

*dunni*, *cardini*, *Wolbachia*, parthenogenesis, Saint Lucia, Antilles, host-parasite

## INTRODUCTION

Interactions between insects and their parasitoids are ubiquitous across terrestrial ecosystems, and have been postulated as critical, but underappreciated, drivers of ecological speciation in insect groups [1, 2]. Yet compared to other ecological interactions, considerably less attention is paid to parasitism as a driver of insect speciation, in part because of the challenges of identifying often cryptic parasitoid species. Molecular-based identification methods have rapidly revolutionized species identification, but issues persist for many parasitoid lineages [3]. Most critically, while parasitoids are commonly acknowledged as one of the most speciose groups of organisms, they are persistently under-described and under-represented in insect conservation efforts [4-6]. As such, more effort to find and describe the ecology and unique biology of new parasitoid species is needed to better understand the pattern and scope of biodiversity on earth.

Island species groups have been attractive systems for the study of speciation events and adaptive radiations since as far back as Darwin (1859), who recognized the immense number of endemic species on archipelago chains. Island archipelagos offer unique opportunities to study peripatric speciation following discrete patterns of colonization between single islands in an archipelago chain [7]. *Drosophila* species groups have been of particular interest for studying patterns of island colonization and speciation. Indeed, the Hawaiian *Drosophila* group represents one of the most well studied examples of island radiations – home to at least 500 species and research spanning decades [8-10]. While Hawaiian *Drosophila* are the most intensely studied group, several other island clades of *Drosophila* have received research attention (e.g., *sechellia-simulans* [11, 12]). Another such clade is the *D. dunni* group – a subgroup of the larger *D. cardini* group that is endemic to the Caribbean islands, particularly the Lesser Antilles [13, 14]. This subgroup is characterized by species or subspecies with distinct abdominal pigmentation endemic to just one or two major islands in this archipelago – frequently just 25-50 km apart. As such, this group represents an excellent model for the study of peripatric speciation and morphological evolution across archipelagos [12, 14]. Despite years of appreciation for the *dunni* group as a model, little is known about the parasitoid complex of these Caribbean flies.

To better understand the ecology of *dunni* group *Drosophila,* and to investigate their potential parasitoids, we (JSD, SEM, WDT) conducted an expedition to the islands of the Lesser Antilles February through May of 2019. We traveled among the islands aboard the sailing vessel Sea Salt (a 42-foot Fountaine Pajot Catamaran) which also served as a mobile laboratory. We describe here results with collected flies and associated parasitoids from the island of Saint Lucia. Other results of our expedition will be described elsewhere. We describe a new species of *Drosophila* parasitoid: *Leptopilina n. sp.* Buffington, Lue, Davis & Tracey sp. nov. (Hymenoptera: Figitidae). *Leptopilina n. sp.* appears to be endemic to the Lesser Antilles and we find that it has high virulence against *dunni* group flies, particularly *Drosophila antillea* also collected on Saint Lucia. To our knowledge, this is the first host-parasitoid association recorded for *cardini* and *dunni* group *Drosophila.* We find that the wasps reproduce via thelytokous parthenogenesis (the asexual reproduction of females) and that this is caused by infection with a bacterium: a parthenogenesis-inducing *Wolbachia* symbiont. We report *de novo* genome assemblies for *Leptopilina n. sp.* and its *Wolbachia* strain, hereinafter "*w*Lmal". *Leptopilina n. sp.* has the largest *Leptopilina* genome reported to-date (~1Gbp) and will serve as a useful resource for understanding the evolution of *Drosophila*-*Leptopilina* relationships in the Caribbean islands, island speciation events, and parasitoid genome evolution more broadly.

## METHODS

### Collection: Field sites and field rearing

After sailing from Wallliabou Bay of Saint Vincent and the Grenadines, we moored the research vessel and operated out of Soufrière and Marigot Bays of Saint Lucia. Verbal permission for collection of *Drosophila* and their parasitoids was obtained in a February 2019 meeting with Dr. Hannah Romain of the Saint Lucia Ministry of Agriculture. Sampling was conducted in 2019 on Saint Lucia and leveraged baited trapping with either banana or calabasa, a locally available cucurbit fruit (likely *Cucurbita moschata*). Calabasa and banana traps contained chopped fruit mixed with water and baker's yeast that had been kept in a closed bucket for one or more days to ferment. The Calabasa bait was chosen based on communication with Hope Hollecher who found it to be particularly attractive to *dunni* group *Drosophila.* Traps were set at two separate sites from which flies and wasps in this study derive. The first site, known

as STL3 was placed on 24 February 2019 with a total of 2 calabasa traps on a short trail leading off the main road near Ladera resort and Hotel Chocolat (GPS: lat 13.83, lon -61.05, elevation 289.6m, Figure 1). The second site, known as STL6 was placed on 26 February 2019 with a total of eight traps (5 calabasa, 3 banana) along Barre d'Isle trail (GPS: lat 13.924, lon -60.959, elevation 308.7m, Figure 1). Each trapping site was checked daily for three days and insects were collected off the fruit each time. Flies were collected using either cardboard mailing tubes for vertical trapping or mouth aspiration, and wasps were collected by mouth aspiration while they searched for larvae in the bait.

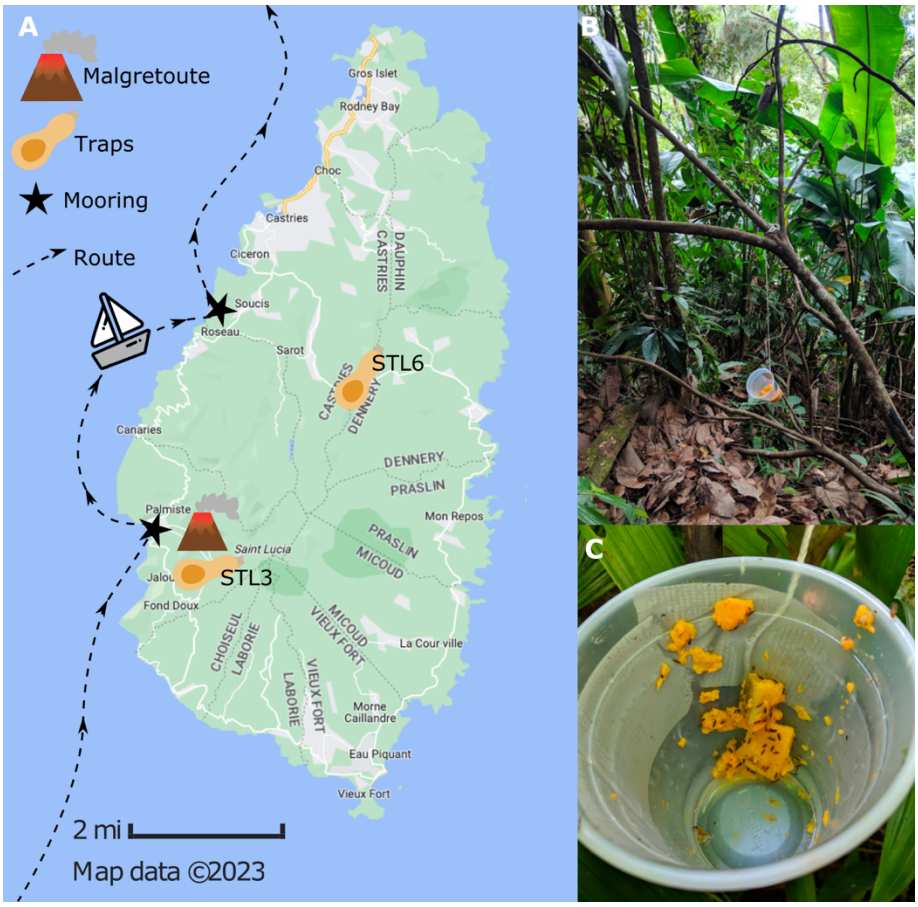



**Figure 1. Collections on Saint Lucia: field notebook entry from JSD on 1 March 2019:** "*...a 40-minute brisk hike, in which I found 0 wasps in the traps - [bummer]. Until I remembered I had set out one more trap off the path a little that I had not checked yet. And [nevertheless], my lucky [telson] finds the wasp we needed sitting in this cup. I managed to nab it and was pretty excited leaving here.*" **(A)** Map of Saint Lucia (GoogleMaps 2023), annotated with (1) locations of the STL3 and STL6 trap placements, (2) the route and mooring locations (Soufrière in the south, and Marigot in the north) of the Sea Salt, and (3) Malgretoute: the location of the La Soufrière Volcano. **(B)** The STL6 calabasa trap. **(C)** The inside of a calabasa trap.

126 **Collection: Colony establishment and maintenance**

127 After collection, flies and wasps were transferred to individual rearing vials with instant fly media (Formula
128 4-24® Instant Drosophila Medium, Blue; Carolina Biological Supply Company). Isofemale lines of
129 *Drosophila antillea* and *Drosophila willistoni*, were initiated using flies collected at STL3 (see results). A
130 single wasp was collected from STL6 and was left in a vial with developing STL3 *Drosophila willistoni*
131 larvae. This vial was then shipped back to Indiana University Bloomington under USDA APHIS
132 Importation Permit Number P526P-19-01355. Since 2019, wasps have been maintained continuously on
133 host cultures of *D. willistoni* on Bloomington fly food medium and further reared in IU USDA regulated
134 containment facility number 5035.

135

136 **Taxonomy: Imaging**

137 Voucher specimens were sent to two of us (CHL and MB) for identification. These specimens were
138 compared with specimens deposited in the USNM as well as those on loan from Goran Nordlander
139 (Uppsala Agricultural University, retired). Additionally, notes from Yves Carton were shared with MB, and
140 his work with G. Nordlander in the mid 1980's. These notes revealed an undescribed *Leptopilina* from
141 Guadeloupe that matched the species described herein. We are therefore including these older
142 specimens in the description. Images were acquired using a Hitachi 3000 desktop Scanning Electron
143 Microscope (SEM), after a 120nm coating of Au-Pd from a Cressington sputter-coater. Light micrographs
144 were shot using a Macropod 3D Pro camera setup. All type specimens collected in this study are housed
145 in the USNM (registration numbers below); additional types, examined by G. Nordlander, reside in the
146 Uppsala Agricultural University collection overseen by G. Nordander. Terminology for these descriptions
147 follows Lue et al. (2016) [15].

148

149 **Taxonomy: Systematics of *Leptopilina***

150 Lue et al. (2016) [15] provided a detailed description on the species limits of *Leptopilina*. Species in this
151 genus are common throughout the Holarctic Region and have recently been investigated for their
152 biological control potential of pestiferous Drosophilidae [16, 17]. Some 29 species are currently
153 recognized worldwide [15]. While most described species are endemic to the Holarctic Region, many

154 undescribed species remain in collections, awaiting description. A relatively common trait among
155 Neotropical *Leptopilina* are polychromatic antennal segments, specifically black/brown, cream, and
156 orange-colored segments. These traits are either rare, or wholly missing, from the Paleotropics and
157 Australia (Buffington, Lue, pers. obsv.). This is mentioned here as the new species, herein described, has
158 rather unique color patterns among the antennal segments. This character makes this species relatively
159 straightforward to diagnose from other *Leptopilina*.
160
161 **Biology: Virulence assessment**
162 To test the virulence of *Leptopilina n. sp.* on *Drosophila* host species, infection vials were established at a
163 wasp-to-larvae ratio of 1:10 (typically 5 female wasps and 50 drosophila larvae). Two isofemale lines from
164 two *Drosophila* species that were collected from the island of Saint Lucia were assessed: *Drosophila*
165 *willistoni* (line STL3 iso3) and *Drosophila antillea* (line STL3 iso1). To collect the age-matched larvae, 20-
166 30 male and female adult flies were placed in embryo collection cages. The embryo collection plates (60
167 x 15mm) contained 5mL of *Drosophila* banana food medium (Archon Scientific) and a small amount of
168 yeast paste (active dry yeast mixed with water). The embryo collection cages were maintained in an
169 incubator set at 25°C and 75% humidity on a 12h/12h light/dark cycle. Embryos from a restricted 12-hour
170 egg-laying window were allowed to develop in the incubator for 3 days until they reached the size of 2$^{nd}$
171 instar larvae. Larvae were carefully gently collected using extreme caution so as to not damage them.
172 Fifty larvae were placed into individual vials containing *Drosophila* banana food and a small cotton dental
173 plug that was inserted into the center of the food. The cotton plug was necessary to provide a place for
174 pupariation for the *D. antillea* species (which avoids pupariation on the side of the culture vials). Five
175 naïve *Leptopilina n. sp.* female wasps were placed into each infection vial and were allowed to infect the
176 larvae for 24 hours. During the infection period, the vials were closed with Flugs (Genesee Scientific
177 cat.no.49-102) dampened with honey and water (1:1 mixture) to ensure that the wasps had access to
178 food. After the infection period, the wasps were removed, and the vials were incubated as described
179 above. Uninfected mock control vials were treated in the same way but were not exposed to wasps. The
180 vials were assessed daily for the emergence of adult insects. Adult flies eclosed after 10-14 days, and
181 adult wasps eclosed after 25-30 days. Any newly eclosed adult flies and wasps were counted and

182 removed from the vials. The difference between the total number of adults and the initial number of larvae
183 in each vial accounts for larval mortality.
184
185 **Biology: Western blots for *Wolbachia* detection**
186 Active *Wolbachia* infection was assessed with western blot following published protocols [18]. Positive
187 and negative controls included individual *Drosophila melanogaster* from previously validated *Wolbachia*-
188 infected and -uninfected stocks (RRID: BDSC_145) [19]. Individual insects were homogenized with a
189 pestle in 1.7 ml microcentrifuge tubes containing lysis buffer (150mM NaCl, 1% Triton X-100, 50mM
190 TrisHCl (pH8) containing HALT protease inhibitor cocktail (Thermo Scientific) and 5 mM EDTA).
191 Homogenates were pelleted via centrifugation (8,000Xg for 1 minute) and the supernatant was incubated
192 for 5 minutes at 95°C in Laemmli sample buffer with 5% β-mercaptoethanol (Bio-Rad). Proteins were
193 separated via SDS-PAGE on 4–20% Tris-Glycine gels (NuSep) in 1X Tris/Glycine/SDS running buffer
194 (Bio-Rad) alongside a PageRuler prestained protein ladder (Thermo Scientific), transferred to a PVDF
195 membrane in Tris-Glycine transfer buffer with 15% methanol for 3 hours at 40v, and blocked for 5 minutes
196 in Starting Block T20 (Thermo Scientific). The membrane was co-incubated in primary antibodies
197 overnight at 4°C. After washing, the blot was incubated with the secondary antibody anti-mouse:HRP
198 (Invitrogen) at 1:5000. Finally, HRP was detected with SuperSignal West Pico Chemiluminescent
199 Substrate (Thermo Scientific). Primary antibodies included (1) mouse monoclonal Anti-Wolbachia Surface
200 Protein (WSP), NR-31029 from BEI Resources, NIAID, NIH at 1:10,000, and (2) mouse anti-actin
201 monoclonal at 1:20,000 (Seven Hills Bioreagents) as a loading control.
202
203 **Biology: Determining reproductive mode**
204 To test whether the observed parthenogenesis was induced by infection with a bacterial symbiont,
205 *Leptopilina n. sp.* female wasps were fed with the antibiotic rifampicin mixed into honey (100 mg of
206 rifampicin powder (Sigma-Aldrich cat.no. R3501-250MG) with 1 mL of honey) and reproductive biology
207 was assessed following standard protocols [20]. To feed the wasps, a Flug was dipped into water to
208 dampen one side, onto which a small amount of the rifampicin-honey mixture was spread (approx. 10-20
209 μL). A fresh Flug with rifampicin-honey was replaced once a day for six days. To prepare vials of fly

210 larvae, adult *Drosophila* were allowed to seed vials for 5 days, after which the adults were removed. After
211 the six days of rifampicin treatment, the treated wasps were placed into the vials with fly larvae and
212 allowed to infect as many larvae as possible. Wasp offspring eclosed after 25-30 days and all female and
213 male offspring were collected and counted. Rifampicin treatment trials were performed with larval hosts
214 from fly species that were collected on the island of Saint Lucia, either *D. melanogaster* or *D. willistoni*.
215 Regardless of the host species, the sex ratio of the wasp offspring in each generation was always almost
216 all female or almost all male (see results, Figure 5), and therefore the data from the trials with different
217 host species were combined. The treatment conditions of each generation were as follows: $G_1$ unmated
218 females received 6 days of rifampicin treatment. The $G_2$ offspring were almost all female and also
219 received 6 days of rifampicin treatment, after which they produced $F_0$ offspring. In order to cross
220 *Wolbachia* depleted males and females together, a separate generation of $G_1$ females was treated with
221 rifampicin to produce $G_2$ females that would eclose at the same time as the $F_0$ males. These $G_2$ females
222 then received 6 days of rifampicin treatment and were then mated to $F_0$ males. Subsequent generations
223 ($F_1$ - $F_5$) were sexed and propagated by attempting sibling mating (when possible, see results) without
224 further rifampicin treatments.
225
226 **Genomics: Oxford Nanopore library preparation and sequencing**
227 DNA was extracted from a pool of ~100 adult female wasps using a Gentra PureGene Tissue Kit
228 (Qiagen). 3 ug of DNA was end prepped using NEBNext FFPE Repair Mix (M6630), NEBNext Ultra II End
229 repair/dA-tailing Module (E7546), and NEBNext Quick Ligation Module (E6056) and subsequently library
230 prepped using the ONT supplied SQK- LSK110 kit. Sequencing took place on an Oxford Nanopore
231 Minion over 3 days with nuclease wash followed by reloading after 24 hours. The sequencing computer
232 was built with an AMD Ryzen 3900x processor with 12 threads, 24 cores, 64 Gb RAM and a 1 Tb SSD,
233 and a GeForce 2080Ti with 4352 CUDA cores running Ubuntu Linux 20.04. We monitored the CUDA
234 cores with nvtop (https://github.com/Syllo/nvtop). Fastq files were generated from the Oxford Nanopore
235 Minion instrument and called with Guppy v6.1.5. We used the GPU enabled version of guppy in concert
236 to enable live basecalling with the fast base calling model and for post-hoc base calling with the super

237 (sup) accuracy model called via Minknow v22.05.5. All resulting fastq.gz files were concatenated and
238 used for further processing.
239
240 **Genomics: Illumina sequencing**
241 Total DNA was extracted from a pool of ten adult wasps using the DNeasy blood and tissue kit (Qiagen).
242 Sequencing library preparation was performed with the NEBNext Ultra II DNA library preparation kit (New
243 England Biolabs) according to the manufacturer's protocols including 10 cycles of PCR amplification. The
244 resulting library was subjected to 150-bp paired-end sequencing on the Illumina NextSeq 500 platform at
245 the Indiana University Center for Genomics and Bioinformatics (Bloomington, IN) to generate
246 181,561,108 read pairs. To aid in gene annotation we also sequenced wasp transcriptomes. Total RNA
247 was extracted from a flash-frozen, mixed stage pool of 10 wasps (adults, pupae, larvae) using the
248 Monarch® Total RNA Miniprep Kit (New England Biolabs) including the DNase treatment step. An RNA-
249 Seq library was prepared with the Illumina TruSeq Stranded mRNA Library Prep Kit, including rRNA
250 removal with oligo-dT beads, and sequenced on a NovaSeq with S4 chemistry at the University of
251 Minnesota Genomics Center to generate 64,677,726 150-bp paired-end reads.
252
253 **Genomics: Nuclear genome assembly, curation, and quality control**
254 All reads greater than 5kb were filtered with SeqKit v2.5.1 [21], assembled with Flye v2.9 [22], and the
255 assembly was polished with Medaka v1.5.0 (https://github.com/nanoporetech/medaka). Haplotigs were
256 purged with PurgeHaplotigs v1.1.2 [23]. Pilon v.122 [23] was used for polishing: first WGS Illumina reads
257 were mapped to the long-read assembly with bwa v0.7.17 [24], alignment files were converted and sorted
258 with samtools v1.10 [25], and finally pilon was called for polishing to generate an updated assembly. Four
259 rounds of pilon polishing were performed, each time mapping the short reads to the most recent pilon
260 output prior to pilon correction. Manual curation of the assembly and identification of cytoplasmic
261 genomes was informed by results derived from running blobtools [26]. We discarded contigs that were
262 deemed anomalous for the following reasons: (1) less than 5,000 bp in length with no BUSCOs (because
263 >5k reads were used during assembly, these are very likely to be chimeric and/or haplotigs), or, (2)
264 having blast hits to non-arthropod taxa across the length of the contig, unusual sequencing

265 coverage(<15X; >800X), and unusual GC content (outside of 23-33%). Finally, we checked the previously
266 purged contigs and recovered those that appeared to encode for *Leptopilina* rRNAs. Assemblies were
267 assessed with BUSCO v.5.3.2 [27] against the hymenoptera_odb10 database in genome mode ('-m
268 genome').
269
270 **Genomics: Methylation**
271 Nanopore runs were base called with guppy v6.3.8 using model
272 "dna_r9.4.1_450bps_modbases_5mc_cg_sup" to call 5-methylcytosines in a CpG context only. Aligned
273 bam reads containing modified base information were converted into bed format with modbam2bed, with
274 strict parameters on canonical and modified base probabilities where bases are only called as canonical
275 or modified if they have a greater than 95% probability estimate. Parameters: modbam2bed -m 5mC -t 32
276 -e -p total.modmapped --aggregate --cpg dtwasp-final-curated.fa total.modmapped.bam -a 0.05 -b 0.95 >
277 total.modmapped.bed". Global methylation was calculated by an awk script.
278
279 **Genomics: Mitogenome**
280 Mitogenome annotation with MITOS2 [28] and manual rearrangement to start the circular mitogenome at
281 Cox1 (as is convention) was done as in [29]. MITOS2 parameters included the RefSeq 63 Metazoa
282 reference and the invertebrate mitochondrial translation code. Manual curation was facilitated by
283 comparison to published mitochondrial genomes from *Leptopilina boulardi* (KU665622.1) and *Leptopilina*
284 *syphax* (MT649407.1). A phylogeny of *Leptopilina* was constructed based on COX1 ("COI") sequences
285 available from the DROP database [3]. A subset of the available *Leptopilina* sequences were selected
286 based on (1) sampling across the genus, (2) limiting the total number of sequences for any given species,
287 (3) length of the barcoding region sequenced, and (4) checking for open reading frame. In total 55
288 sequences were used for phylogenetic reconstruction. These include an outgroup (*Ganaspis kimorum*),
289 previously sequenced barcodes for the *Leptopilina n. sp.* STL6 colony [3], and the COI sequence derived
290 from the mitogenome assembly. Nucleotide sequences were aligned with MAFFT v7.511 [30] using the L-
291 INS-i alignment algorithm and a five point gap open penalty (-op 5). Comparison to the translated amino
292 acid alignment verified that codon relationships were maintained and we did not need to back-translate an

amino acid alignment. Ends of the nucleotide alignment were trimmed such that all sequences were represented at all positions. Phylogenetic reconstruction was then performed with IQtree v1.6.11 [31] using a pre-analysis model optimization step to select the final model (here, TIM3+F+I+G4) plus 1000 “ultrafast” bootstraps.

**Genomics: Repeat identification**

Repetitive DNA content was estimated using RepeatModeler2 [32] using the curated Dfam database release 3.5 July 2021 [33], combined with the Repbase RepeatMasker database downloaded on October 26, 2018. RepeatMasker was run with the newly identified library to annotate the assembly with the following parameters: RepeatMasker v4.1.2-pl -pa 24 -s [sensitive mode] [using rmblast 2.11.0+] -lib ultimatefamilies.fa ultimateGenome.fasta. Each repeat library was also mapped against all other species genomes with the following parameters: RepeatMasker v4.1.2-pl -pa 24 -s [sensitive mode] [using rmblast 2.11.0+] -lib families.fa genome.fasta.

**Genomics: Gene annotation and ortholog clustering**

Gene models were predicted with GeMoMa v1.9 [34] and leveraged three lines of evidence: (1) RefSeq annotations for *Leptopilina heterotoma* (GCF_015476425.1) (2) RefSeq annotations for *Leptopilina boulardi* (GCF_019393585.1) and (3) RNA-seq reads derived from the mixed pool of wasps. RNA-seq reads were mapped to a hard masked version of the reference genome using STAR v2.5.3a [35]. GeMoMa was called with the following additional parameters: tblastn=TRUE, AnnotationFinalizer.n=false, AnnotationFinalizer.r=SIMPLE, AnnotationFinalizer.t=true, AnnotationFinalizer.p=LMAL, pc=true, o=true. To generate annotations for *Leptopilina clavipes* and *Ganaspis kimorum*, we ran the same annotation pipeline on hard-masked assemblies, only without the RNA-seq data. Protein sequences from each genome were clustered into orthologous groups using OrthoFinder v.2.5.4 [36]. For genes represented by multiple transcript variants, only the longest isoform was used in clustering.

321 **Genomics: *Wolbachia* genome**

322 A single contig was identified to be a *Wolbachia* genome, based on size, circularity, and Blobtools results.
323 Genome completeness was assessed with BUSCO v5.3.2 [27] against the rickettsiales_odb10 database
324 in genome mode ('-m genome'). Genome annotation and phylogenetics were performed by running the
325 *Wolbachia* Phylogeny Pipeline (WHOP; https://github.com/gerthmicha/WHOP [37]). In brief, the pipeline
326 takes a provided database of bacterial genomes (*Wolbachia* strains and Rickettsiales outgroups) plus
327 additional genomes provided by the user, annotates them with Prokka v1.14.6 [38], clusters protein
328 sequences into orthogroups with OrthoFinder v2.5.4 [36], aligns the single-copy orthologs with MAFFT L-
329 INS-i v7.487 [30], removes recombining genes with PhiPack v1.1 [39], and then concatenates the
330 alignments prior to phylogenetic reconstruction with IQtree v2.2.3 [31]. IQtree was first run with a pre-
331 analysis model optimization step (-m LG+I+G4 -pre -redo -fast), followed by final reconstruction with the
332 selected model and 1000 bootstrap replicates. The *w*Lmal genome was re-oriented to start at *dnaA* and
333 proksee (https://proksee.ca/ [40]) was used for visualization. Prophage regions were identified with
334 VirSorter2 v.2.2.4 [41], and Phigaro v.2.3.0 [42], and other mobile elements were identified with
335 mobileOG-db v.1.0.1 [43], all implemented in proksee with default parameters. To aid in manual
336 annotation of *w*Lmal we leveraged the orthogroup relationships between *w*Lmal and *w*Mel generated by
337 WHOP to check for specific proteins. Additionally, we used blastp and tblastn with default parameters to
338 query against the protein sequences or genome, respectively, for a select number of functionally relevant
339 *Wolbachia* proteins not found in *w*Mel. All manual annotation queries and matches are in Supplemental
340 Tables S5-6.

341

342 **Data visualization**

343 Data visualization was carried out in R version 4.1.3 [44] and leveraged Paul Tol's "Medium-contrast
344 qualitative colour scheme", (R: khroma [45]). Newick files from phylogenetic reconstructions were
345 visualized in FigTree v1.4.4 (https://github.com/rambaut/figtree/) and annotated in Inkscape. Proksee
346 (https://proksee.ca/ [40]) was used for genome visualization, and the r package 'gggenes' v.0.5.1 was
347 used to plot gene models.

348

## RESULTS

### Collections on Saint Lucia

Banana and calabasa fruit traps were used to collect *Drosophila* and their associates on Saint Lucia in 2019. The calabasa traps at STL3 yielded six female *Drosophila antillea* and one female *Drosophila willistoni.* Isofemale lines of *D. antillea* and *D. willistoni* were established from single females that originated from the STL3 collection site. At site STL6, banana traps contained a total of 12 *melanogaster*-like flies and 1 *repleta* group fly. Calabasa traps contained 4 *melanogaster*-like flies, 4 *repleta* group flies and 20 *dunni*-group flies. Wasp "STL6" was collected from a calabasa trap on the morning of 1 March 2019: a clear day with a high temperature of 27C (Figure 1). The STL6 wasp was placed in a vial with developing *Drosophila willistoni* (from the STL3 site, see above) to initiate a culture, and then shipped back to Indiana University where they have been maintained since (see methods).

### *Leptopilina n. sp.* Buffington, Lue, Davis & Tracey sp. nov.

**Species Diagnosis**. *Leptopilina n. sp.* is distinguishable from other *Leptopilina* species by a number of characters, including: flagellomere colors, in which F1-F4 are honey-brown, F5-F10 are dark brown, and F11 is yellow (Figure 2A); the typical flagellomere color in *Leptopilina* do not match the unique combination here. Latero-posterior margin of pronotal plate is with elongate, golden setae that reach the anterior margin of the mesoscutum (Figure 2B (LM) and Figure 3A-B (SEM)). No other known *Leptopilina* have setae so long off the pronotal plate (Figure 2B); other species have hairs here, but not so long. Members of the *clavipes* group *sensu* [15] tend to also have sparse, scattered setae over the mesoscutum, whereas *Leptopilina n. sp.* is entirely glabrous (Figure 3A-B).

### Morphological Description

Coloration with head, mesosoma, metasoma black to dark brown, legs light brown. Sculpture on vertex, lateral surface of pronotum and mesoscutum absent, surface smooth.

*Antenna*. Female antenna composed of scape, pecidel, 11 flagellomeres (F1-F11). F1-F4 sub-cylinderical, slightly expanded distally, smooth, non-claval segments. F5-F11 claval segments, abruptly broader than non-claval segments, distinctly barrel-shaped, with slightly more erect, dense setae; claval segments with placoidal sensillae distributed evenly around each segment. Single campaniform sensillae present ventro-apically on F6-F10 (arrow, Figure 3C; compare to Figure 3D). Articulation between flagellomeres in antenna moniliform, segments distinctly separated by narrow neck-like articulation. Male antenna composed of 13 flagellomeres, F1 as long as F2, slightly curved and excavated distally; campaniform sensillae on all flagellomeres.

*Head*. In anterior view, broadly triangular. Pubescence on head sparse, single setae scattered over face. Sculpture along lateral margin of occiput absent. Gena (measured from compound eye to posterolateral margin of head) short, ratio of length of gena to length of compound eye in dorsal view <0.3. Sculpture of gena absent, smooth. Lateral margin of occiput evenly rounded, not well defined. Occiput (except extreme lateral margin) smooth. Carina issuing from lateral margin of postocciput absent. Ocelli small, ratio of maximum diameter of a lateral ocellus to shortest distance between lateral ocelli 0.2-0.4. Anterior ocellus far from posterior ocelli, clearly separate anterior ocellus to anterior margins of posterior ocelli. Relative position of antennal sockets intermediate, ratio of vertical distance between inner margin of antennal foramen and ventral margin of clypeus to vertical distance between anterior ocellus and antennal rim 2.0-4.0. Median keel absent. Vertical carina adjacent to ventral margin of antennal socket absent; facial sculpture absent, surface smooth. Facial impression absent, face flat. Antennal scrobe absent. Anterior tentorial pits small. Vertical delineations on lower face absent. Ventral clypeal margin laterally, close to anterior mandibular articulation, straight. Ventral clypeal margin medially straight, flat, with 4-6 setae overhanging base of mandibles. Clypeus smooth. Malar space adjacent to anterior articulation of mandible evenly rounded, smooth. Malar sulcus present, composed of single groove. Eye removed from lateral ocelli, ratio of distance between compound eye and posterior mandibular articulation to distance between posterior ocellus and compound eye <1.2. Compound eyes, in dorsal view, moderately protruding from the surface of the head. Pubescence on compound eyes absent. Orbital furrows absent. Lateral frontal carina of face absent. Dorsal, posterior aspect of vertex smooth. Hair

punctures on lateral aspect of vertex absent. Posterior surface of head deeply impressed around postocciput.

*Mouthparts*. Apical segment of maxillary palp with pubescence, consisting of one long erect setae. Apical seta on apical segment of maxillary palp longer than twice length of second longest apical seta. Maxillary palp composed of four segments. Last two segments of maxillary palp (in normal repose) straight. Apical segment of maxillary palp about 1.5 times as long as preceding segment. Left mandible with three teeth; scattered setae dorsally.

*Mesosoma.* Long auburn-golden setae present, originating from anterior inflection of pronotum, extending to anterior margin of mesoscutum; rest of pronotum glabrous (Figure 3A-B). Anteroventral inflection of pronotum narrow. Pubescence on lateral surface of pronotum absent. No ridges present posterior to pronotal plate. Anterior flange of pronotal plate distinctly protruding anteriorly, completely smooth. Ridges extending posteriorly from lateral margin of pronotal plate absent. Lateral pronotal carina absent. Crest of pronotal plate absent. Dorsal margin of pronotal plate (in anterior view) spatulate. Submedian pronotal depressions open laterally, deep. Lateral margin of pronotal plate defined all the way to the dorsal margin of the pronotum. Width of pronotal plate narrow, not nearly as wide as head.

Mesoscutal surface convex, evenly curved. Sculpture on mesoscutum absent, entire surface smooth, shiny, with sparse long hairs. Notauli absent. Median mesoscutal carina absent. Anterior admedial lines absent. Median mesoscutal impression absent. Parascutal carina nearly straight.

Mesopleuron entirely smooth. Subpleuron entirely smooth, glabrous. Lower pleuron entirely smooth, glabrous. Epicnemial carina absent. Lateroventral mesopleural carina present, marking abrupt change of slope of mesopectus. Mesopleural triangle absent. Subalar pit not visible. Speculum absent. Mesopleural carina present, complete, composed of one complete, uninterrupted carina. Anterior end of mesopleural carina inserting above notch in anterior margin of mesopleuron; posterior end terminates at setal pit of lower metapleuron.

Dorsal surface of scutellum foveate-areolet (Figure 3E). Circumscutellar carina present, complete, delimiting dorsal and ventral halves of scutellum. Posterior margin of axillula marked by distinct ledge, axillula distinctly impressed adjacent to ledge. Latero-ventral margin of scutellum, posterior to axillula, weakly rugulose. Dorsoposterior part of scutellum rounded. Transverse median carina on scutellar plate absent. Dorsal part of scutellum entirely areolate. Scutellar plate, in dorsal view, medium sized, exposing about half of scutellum, gently foveate with three to four single setae; glandular release pit at posterior end, oval. Scutellar fovea present, two, distinctly margined posteriorly, smooth. Longitudinal scutellar carinae absent. Single longitudinal carina separating scutellar foveae present, elongage, ending past posterior margin of foveae, gently expanding into teardrop-shaped scutellar plate. Postero-lateral margin of scutellum rounded. Lateral bar smooth, narrow.

Posterior impression of metepimeron absent. Metapectal cavity anterodorsal to metacoxal base present, well-defined (Figure 3F, arrow A). Anterior margin of metapectal-propodeal complex meeting mesopleuron at same level at point corresponding to anterior end of metapleural carina. Posteroventral corner of metapleuron (in lateral view) not extended posteriorly. Anterior impression of metepimeron absent. Posterior margin of metepimeron distinct, separating metepimeron from propodeum, with distinct ridge (Figure 3F, arrow B). Anteroventral setal pit of lower metapleuron present, with foamy setae. Subalar area broadened anteriorly, narrowed posteriorly. Prespiracular process present, blunt, lobe-like, polished, at end of polished prespiracular groove; posteroventral corner of prespiracular groove extended to distinct lobe. Dorsellum absent. Anterior impression of metepisternum, immediately beneath anterior end of metapleural carina, present. Pubescence consisting of few hairs on posterior part of metepisternum, few sparse hairs on propodeum, but with distinct posterior swatch of foamy setae at midline of metapleuron.

Pubescence posterolaterally on metacoxae sparse, with a single postero-dorsal button of dense pubescence. Microsculpture on hind coxa absent. Longitudinal ridge on the posterior surface of metatibia absent. Metafemoral tooth present, elongate, with adjacent serrate ridge posteriorly. Ratio of first

460 metatarsal segment to remaining 4 segments less than 10; metatarsus 5 distinctly elongate relative to 2
461 through 4.
462
463 Wing vein M absent. Pubescence of fore wing present, long, moderately dense on most of surface. Apical
464 margin of female fore wing rounded. Rs+M of forewing defined but nebulous at point of origin from basal
465 vein at posterior third. Mesal end of Rs+M vein situated closer to anterior margin of wing, directed
466 towards middle of basalis. Vein R1 forming marginal cell completely. Basal abscissa of R1 (the abscissa
467 between 2r and the wing margin) of fore wing as broad as adjacent wing veins. Coloration of wing absent,
468 entire wing hyaline. Marginal cell of fore wing membranous, similar to other wing cells. Areolet absent.
469 Hair fringe along apical margin of fore wing present, long or very long (Figure 2C).
470
471 Propodeal spurs absent. Lateral propodeal carinae present, not reaching scutellum. Ventral end of lateral
472 propodeal carina reaching nucha, carinae separated from each other, elbowed at midpoint, not parallel.
473 Inter propodeal carinae space lightly setose, underlying surface appearing smooth. Petiolar rim of uniform
474 width along entire circumference. Petiolar foramen removed from metacoxae, directed posteriorly.
475 Horizontal carina running anteriorly from lateral propodeal carina absent, moderately setose. Calyptra, in
476 lateral view, rounded. Propodeum not strongly drawn out posteriorly. Calyptra, in posterior view,
477 dorsoventrally elongate or rounded.
478
479 *Metasoma.* Petiole about as long as wide. Surface of petiole longitudinally costate, ventral keel absent.
480 Posterior part of female petiole abruptly widened. Ventral and lateral parts of petiolar rim broad.
481
482 Setal band (hairy ring) at base of tergum 3 present, interrupted dorsally, ventrally. Tergum 3 indistinct,
483 fused with syntergum. Posterior margin of tergum 3 indistinct, fused with tergum 4 in syntergum. Posterior
484 margin of tergum 4 evenly rounded. Sternum 3 encompassed by syntergum. Sculpture on metasomal
485 terga absent. Syntergum present with terga 3 to 5 fused, ventral margin rounded. Peglike setae on T6-T7
486 absent. Postero-ventral cavities of female metasoma T7 present, glabrous save for few, long setae.

Female postero-ventral margin of T6-T7 straight, parallel. Terebrum and hypopygium (in lateral view) curved, pointing upward. Ovipositor clip present.

**Etymology**. The species epithet refers to locations on Saint Lucia: Malgretoute, a town near the island's active volcano, and the near-by Malgretoute Beach, from which the iconic Saint Lucia Pitons are visible.

**Material examined. Holotype:** Female. Saint Lucia, Barr d'Isle trail, central. lat 13.924, long -60.959, elevation 308.7m. 17.II.2019, Jeremy S. Davis, collected from calabasa bait trap. USNMENT01025555. [1] Actual specimen taken from Indiana University colony derived from the above collection locality.

**Paratypes:** 9 females, 3 males. Same collection data as holotype. USNMENT01557161, USNMENT01025556, USNMENT01025557, USNMENT01025554, USNMENT01557162, USNMENT01557158, USNMENT01448469, USNMENT01448467, USNMENT01932975-USNMENT01932978.

**Other material examined**. 8 females. INRA laboratory, Petit Bourg, Guadeloupe, January 1982. Carton, coll. USNMENT01025775-USNMENT01025782

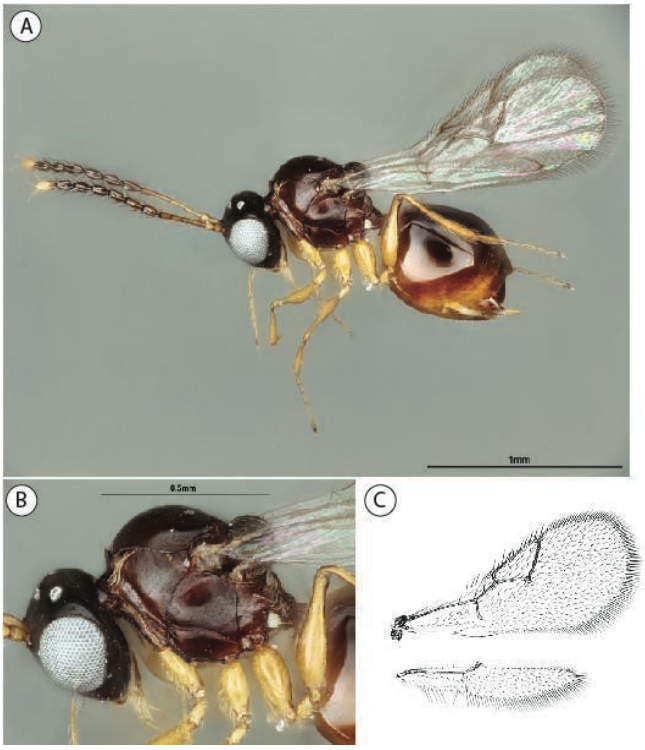



**Figure 2. *Leptopilina n. sp.*. (A)** Lateral view, **(B)** lateral view of mesosoma, and **(C)** wing vein pattern.

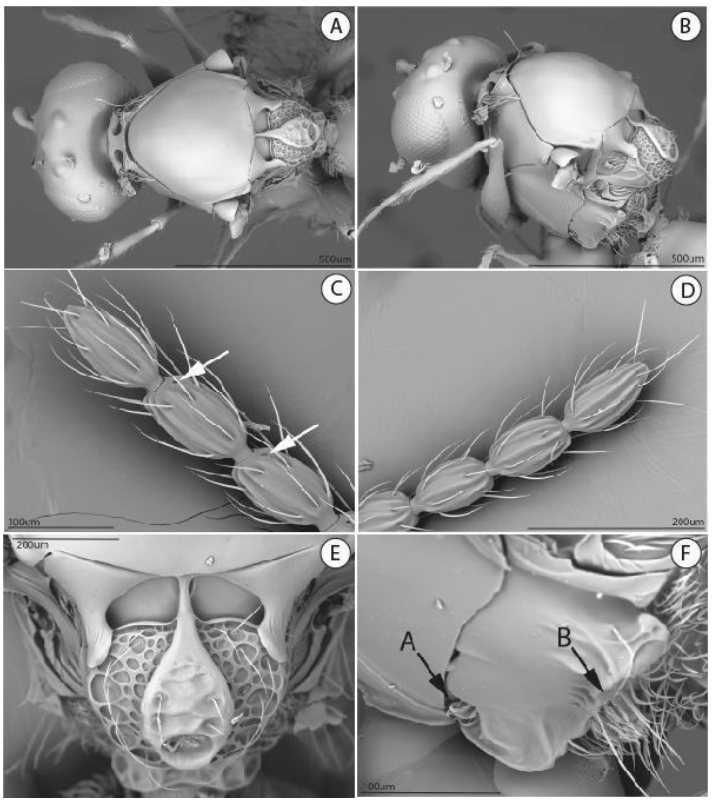



**Figure 3. SEM images of *Leptopilina n. sp.*. (A)** Top view of mesoscutum, **(B)** Lateral-posterior view of mesosoma, **(C)** campaniform sensillae, white arrow, compared to **(D)** no campaniform sensillae, **(E)** scutellum plate, and **(F)** metepimeron.

**<u>*Leptopilina n. sp.* is highly virulent on *D. antillea* flies</u>**

As noted extensively above, our laboratory cultures of *Leptopilina n. sp.* were collected on Saint Lucia. This island is also the only reported location for the *Drosophila antillea* species of the dunni subgroup. To our knowledge, the susceptibility of dunni group *Drosophila* to parasitoid wasps has not been previously investigated. Thus, we wondered if the *D. antillea* flies would be susceptible, or if they were possibly resistant, to infection by a wasp species that is also found on Saint Lucia. We allowed female wasps to oviposit for 24 hours on larval cultures that were derived from isofemale lines of either *D. antillea* or *D. willistoni* (that both originated from the STL3 collection site) and then determined the proportion of wasps and flies that emerged from the infection vials. The results clearly indicated a high degree of virulence of *Leptopilina n. sp.* on *D. antillea* (76.5% ± 5.6% wasp eclosion) relative to *D. willistoni* (20% ± 3.8% wasp eclosion) as host species (Figure 4).

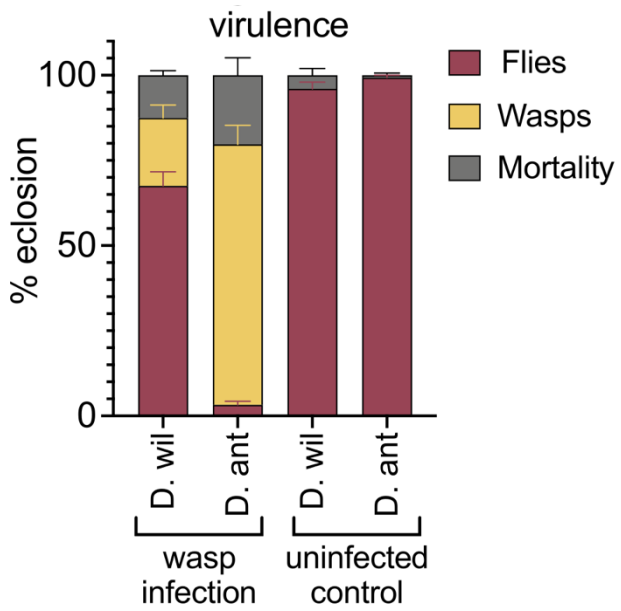



**Figure 4. *Leptopilina n. sp.* shows high virulence on a host fly species that is exclusive to Saint Lucia.** Percentage of adult flies and wasps that eclosed from vials infected by *Leptopilina n. sp.,* and from uninfected control vials. Mortality represents the percentage of larvae that did not develop to adulthood. *L. mal.* is clearly more virulent on *Drosophila antillea* ("D. ant") hosts than on a *Drosophila willistoni* ("D. wil") host strain that was also collected on Saint Lucia. Data are plotted as mean ± SEM; n = 8 infection vials, n = 3 uninfected control vials.

***Wolbachia* mediates obligate thelytokous parthenogenesis in *Leptopilina n. sp.***

While maintaining *Leptopilina n. sp.* wasp lines we noticed that the cultures were primarily female (>95%). And, in one generation of passaging, the cultures became entirely female. Fearing that we would lose the culture, we infected flies with these female wasps in an attempt to obtain haploid male offspring that could be mated back to their mothers to obtain more diploid females. However, the cultures generated by the virgin female wasps were again entirely female. This indicated that this species of wasp was capable of asexually producing females (*i.e.*, thelytokous parthenogenesis). Furthermore, the observation that males were rarely obtained in the cultures was unlikely to be strictly a consequence of the wasps having especially high rates of fertilization (which gives rise to female offspring in arrhenotokous, sexual, haplodiploids). In many parasitic wasp species, including a suite of other *Leptopilina sp.*, thelytokous parthenogenesis is mediated by vertically transmitted bacterial symbionts such as *Wolbachia* (α-proteobacteria: Rickettsiales) [46-48]. We tested for the presence of *Wolbachia* infection by Western blot, targeting a *Wolbachia* specific protein and found that indeed the wasps were infected with this microbe (Supplemental Figure S1).

542

543 To determine if a microbe was responsible for the asexual production of females, we used antibiotic
544 treatment with rifampicin to remove the infection and assess changes in the offspring sex ratio derived
545 from unmated females (Figure 5A). After treating unmated adult females ($G_1$) with rifampicin, they were
546 allowed to parasitize host larvae. Their broods ($G_2$) were almost entirely female (98.6% ± 2.6% female).
547 Critically, *Leptopilina* are largely proovigenic: all their eggs develop and mature during the pupal period
548 [49]. This means that symbiont-mediated effects are likely established much earlier, and treating adults is
549 "too late" (as has been seen in other wasps [50]). Thus, we performed a second generation of rifampicin
550 treatment: $G_2$ rifampicin-treated females produced offspring ($F_0$) that were almost all male (99.7% ± 0.3%
551 male), a clear indicator of symbiont-mediated parthenogenesis.
552

553 We attempted to generate a symbiont-free sexually reproducing line of wasps (Figure 5B) but were
554 unsuccessful. Specifically, symbiont depleted males and treated $G_2$ females were crossed to assess
555 sexual reproduction, but offspring continued to be almost all male ($F_1$-$F_2$, 91.2% ± 1.5% and 98.5% ±
556 0.6% male, respectively), indicating no sexual reproduction (Figure 5C). After antibiotic treatment was
557 discontinued, rare female progeny that were allowed to reproduce produced sex ratios that gradually
558 returned to being almost entirely female ($F_3$-$F_4$, 80% and 100% female, respectively), and unmated
559 females were again able to produce female offspring by parthenogenesis ($F_5$-$F_6$, 98.5% and 100%
560 female, respectively).

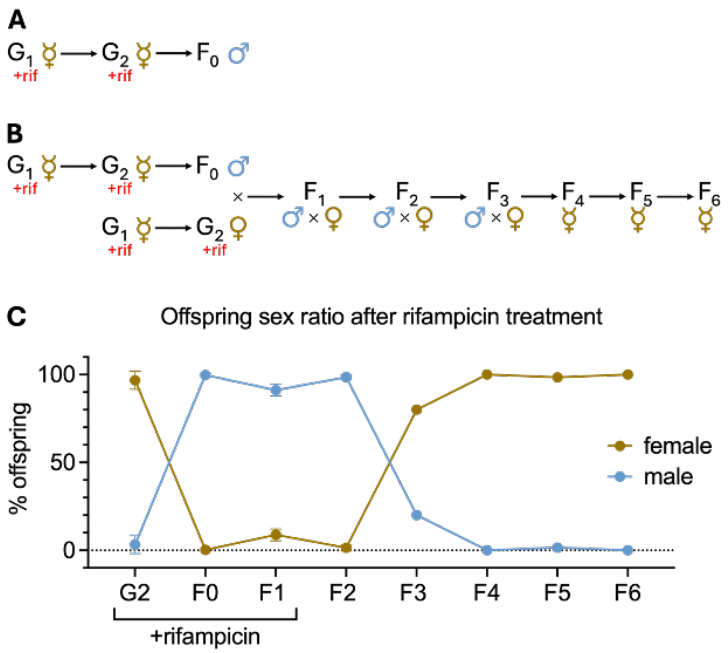



**Figure 5. *Leptopilina n. sp.* reproduces by symbiont-mediated thelytokous parthenogenesis. (A)** Schematic depicting the production of male progeny after two generations of antibiotic treatment ("+rif"), which supports the hypothesis of symbiont mediated parthenogenesis. **(B)** Schematic of antibiotic treatment and crossing scheme to test for sexual reproduction after symbiont depletion, and an attempt to generate a sexual, symbiont free strain. **(C)** Percentage of female and male offspring in each generation. Data is plotted as mean ± SEM. For generations $G_2$ to $F_2$, n = 4-8 infection vials; for generations $F_3$ to $F_6$, n = 1 infection vial.

***Leptopilina n. sp.* genome sequencing and assembly**

We generated a genome assembly for *Leptopilina n. sp.* based on nanopore long read sequencing (Table 1, Supplemental Tables S1-S3). The draft assembly with Flye leveraged all nanopore reads longer than 5,000 bp (Supplemental Table S1) and generated 1,666 contigs totaling just over 1Gbp in total length (Table 1; Supplemental Figure S2). The assembly was then polished with illumina short reads: short-read mapping rates were very high (98.9% of reads mapped). After polishing, separating cytoplasmic genomes, removing haplotigs, purging contaminants, and removing otherwise spurious contigs, the ultimate nuclear genome assembly was 1,408 contigs and ~995Mbp in size with an N50 of >1.8Mbp (Table 1). The longest contig in the assembly was close to 13Mbp in length. Assembly quality was assessed with BUSCO. Just under 90% of the hymenopteran BUSCO proteins were complete, similar to other assemblies in the genus (Table 2). We recovered two cytoplasmic genomes: mitochondrion and *Wolbachia*. Additional low-coverage bacterial sequences that were purged from the assembly belonged to typical associates of the fly food and microbiome (Supplemental Results in File S1 and Supplemental

583 Table S3). No other symbionts were identified in the contigs (e.g., *Cardinium*, *Spiroplasma,* etc [51-53]).

584 Thus, we determine that *Wolbachia* is likely the cause of thelytokous parthenogenesis.

585

586 **Table 1. Assembly statistics for *Leptopilina n. sp.***

| Metric | Draft | Ultimate |
|---|---|---|
| **Contigs** | 1,666 | 1,408 |
| **Length (bp)** | 1,004,830,435 | 994,948,826 |
| **Min length** | 499 | 5,452 |
| **Avg length** | 603,134 | 706,640 |
| **Max length** | 12,918,385 | 12,924,377 |
| **N50** | 1,831,405 | 1,835,757 |
| **%GC** | 29.85 | 29.75 |
| **BUSCO* (Hymenoptera)** | C:89.3%[S:88.8%,D:0.5%], F:2.9%,M:7.8%,n:5991 | C:89.7%[S:89.2%,D:0.5%],F:2.7%,M:7.6%,n:5991 |

587 *Standard BUSCO annotation: Complete BUSCOs (C) [Complete and single-copy BUSCOs (S),
588 Complete and duplicated BUSCOs (D)], Fragmented BUSCOs (F), Missing BUSCOs (M), Total BUSCO
589 groups searched (n)
590

591 **Table 2. Additional genomes used in comparative analyses.**

| Species | Assembly Accession | Size (bp) | Protein Coding Genes | BUSCO* (Hymenoptera) |
|---|---|---|---|---|
| *Leptopilina boulardi* | ZJU_Lbou_2.1 | 354,804,354 | 13,647 | C:91.4%[S:90.9%,D:0.5%], F:1.7%,M:6.9%,n:5991 |
| *Leptopilina clavipes* | ASM185565v1 | 255,343,917 | 13,920 | C:77.8%[S:77.3%,D:0.5%], F:7.3%,M:14.9%,n:5991 |
| *Leptopilina heterotoma* | ASM1547642v1 | 484,694,486 | 14,450 | C:91.1%[S:90.6%,D:0.5%], F:1.9%,M:7.0%,n:5991 |
| *Ganaspis kimorum*[#] | GCA_037103525.1 | 1,025,447,244 | 20,567 | C:89.4%[S:88.8%,D:0.6%], F:2.7%,M:7.9%,n:5991 |

592 * Standard BUSCO annotation: Complete BUSCOs (C) [Complete and single-copy BUSCOs (S),
593 Complete and duplicated BUSCOs (D)], Fragmented BUSCOs (F), Missing BUSCOs (M), Total
594 BUSCO groups searched (n). Hymenoptera dataset used.
595 [#] Previously '*Ganaspis* near *brasiliensis'* (including in genome report [54]), but recently re-assigned [6].
596

597

598

599 **Methylation**

600 We used a methyl-sensitive base-calling model to predict 5-methylcytosines in a CpG context. Across the
601 nuclear, mitochondrial, and *Wolbachia* genomes, fractional methylation was less than 1% (Genome:
602 0.600%, mitochondrion: 0.547%, *Wolbachia*: 0.598%). These estimates likely do not represent low levels
603 of true genomic methylation, rather, they are probably background noise due to error rates inherent to the
604 sequencing and base calling.

605

606 **Mitogenome**

607 We recovered a circular mitochondrial genome (14,490 bp; 20.6% GC; 3,562X sequencing coverage,
608 Figure 6, Supplemental Table S3). Using the barcoding sequence region from the mitochondrial genome,
609 plus representative sequences across the genus, we reconstructed a phylogeny of *Leptopilina sp.* (Figure
610 6A). *Leptopilina n. sp.* sequences were all identical, and formed a monophyletic group more than 10%
611 divergent from the next most closely related species in the dataset, further supporting its designation as a
612 new species. Importantly, *Leptopilina n. sp.* is sister to a clade within the *Leptopilina* genus that contains
613 all other species for which genome sequences are available (*Leptopilina clavipes, Leptopilina boulardi,*
614 and *Leptopilina heterotoma*). Annotation of the mitochondrial genome revealed that gene order was
615 largely conserved as compared to other *Leptopilina* mitochondria. Additionally, we annotated the
616 expected rRNAs and protein coding genes. However, insect mitochondrial tRNAs are often difficult to
617 annotate [55], and in *Leptopilina n. sp.* we were unable to find tRNAs for asparagine (N), alanine (A),
618 aspartic acid (D), pheylalanine (F), serine anticodon-1 (S1), and arginine (R). Related to that, the region
619 between the 3' end of the 16S rRNA (rrnL) and the 5' end of *nad2*, was quite divergent as compared to
620 *Leptopilina boulardi,* in which there is a cluster of tRNAs (including D, F, S1, N, A, plus glutamic acid,
621 glycine, and histidine). It is likely that divergence and/or noncanonical tRNA structures, as is often the
622 case in insect mitochondria, may be precluding a complete annotation.

623

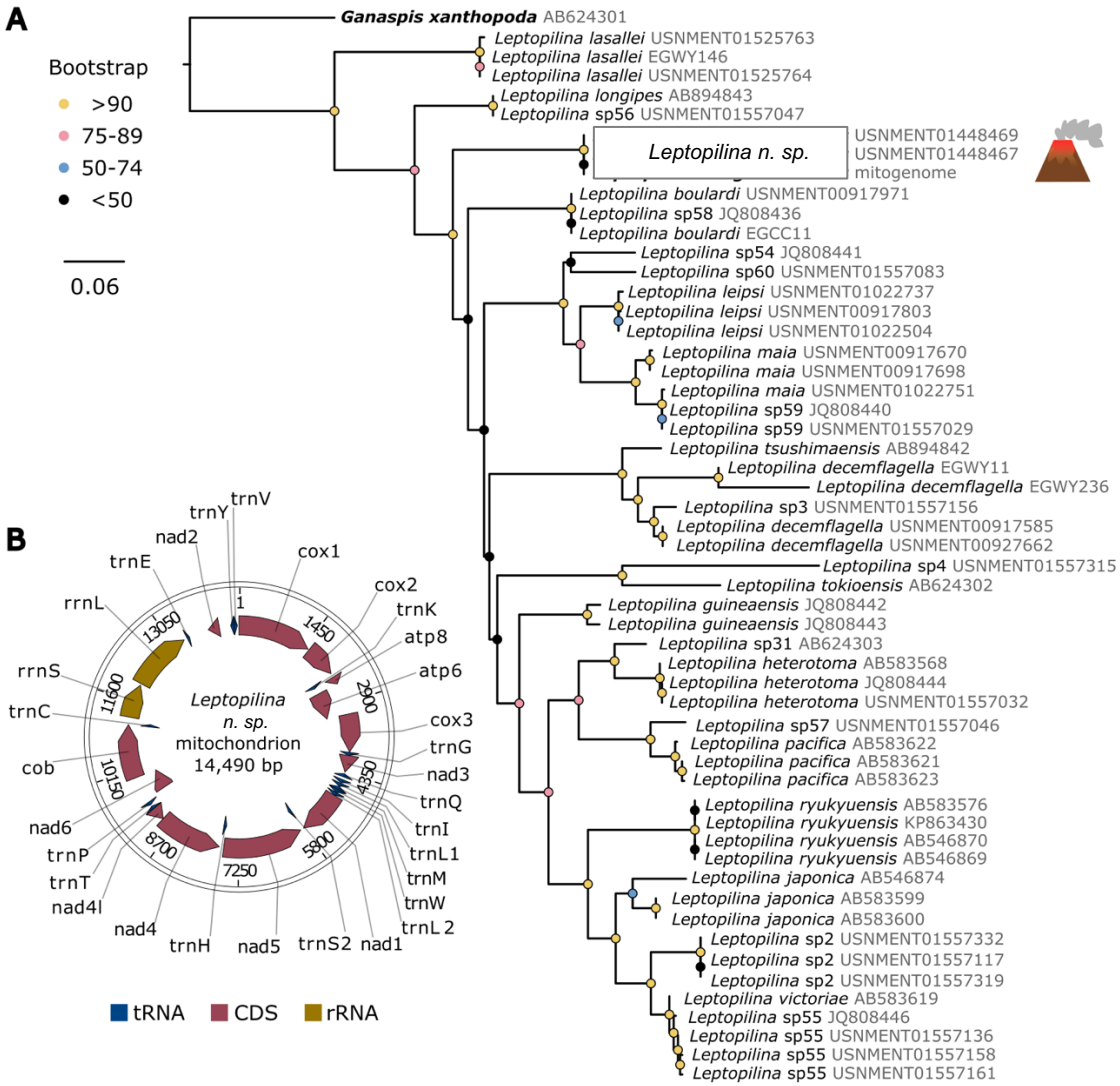



**Figure 6. Mitochondrial phylogeny and genome of *Leptopilina n. sp.*. (A)** Phylogenetic reconstruction of *Leptopilina,* based on 480 nucleotides of Cox1, aligned to maintain codon position relationships. Node colors indicate bootstrap support as indicated in legend. The three *Leptopilina n. sp.* sequences are identical. The two other *Leptopilina n. sp.* sequences are from previously barcoded STL6 specimens [3]. Sequences assigned to provisional species are indicated by 'sp' followed by a number, as per DROP database [3]. Accession numbers are in gray at the end of each leaf label. **(B)** Map of the assembled mitochondrial genome and annotations.

## Gene Annotation

Annotation of the repeat-masked *Leptopilina n. sp.* genome revealed 16,402 genes (Table 3), which is in-line with annotations of closely related species (Table 2). To better understand genome evolution, we clustered protein sequences (n=78,896 in total) of the four *Leptopilina* species plus outgroup species *Ganaspis kimorum* [6, 54, 56-58]. As expected with a small set of closely related species, we identified orthologs for most genes: 97.1% of the genes were clustered into 13,659 orthogroups, each of which

639 contained 5.6 genes on average. 7,266 orthogroups contained genes from all five species, and of those,
640 6,389 orthogroups were strictly single-copy. 1,077 orthogroups were species-specific and only contained
641 in-paralogs.
642
643 **Table 3. Annotation metrics for the *Leptopilina n. sp.* genome.**

| Metric | Value* |
|---|---|
| Number of genes | 16,402 |
| Number of mRNAs | 18,165 |
| Number of exons | 97,163 |
| Number of introns | 78,998 |
| Mean mRNAs per gene | 1.1 |
| Mean exons per mRNA | 5.3 |
| Total gene length | 158,352,048 bp |
| Longest gene | 235,629 bp |
| Mean gene length | 9,654 bp |
| Longest CDS | 76,353 bp |
| Mean CDS length | 1,541 bp |
| Longest exon | 16,278 bp |
| Mean exon length | 288 bp |

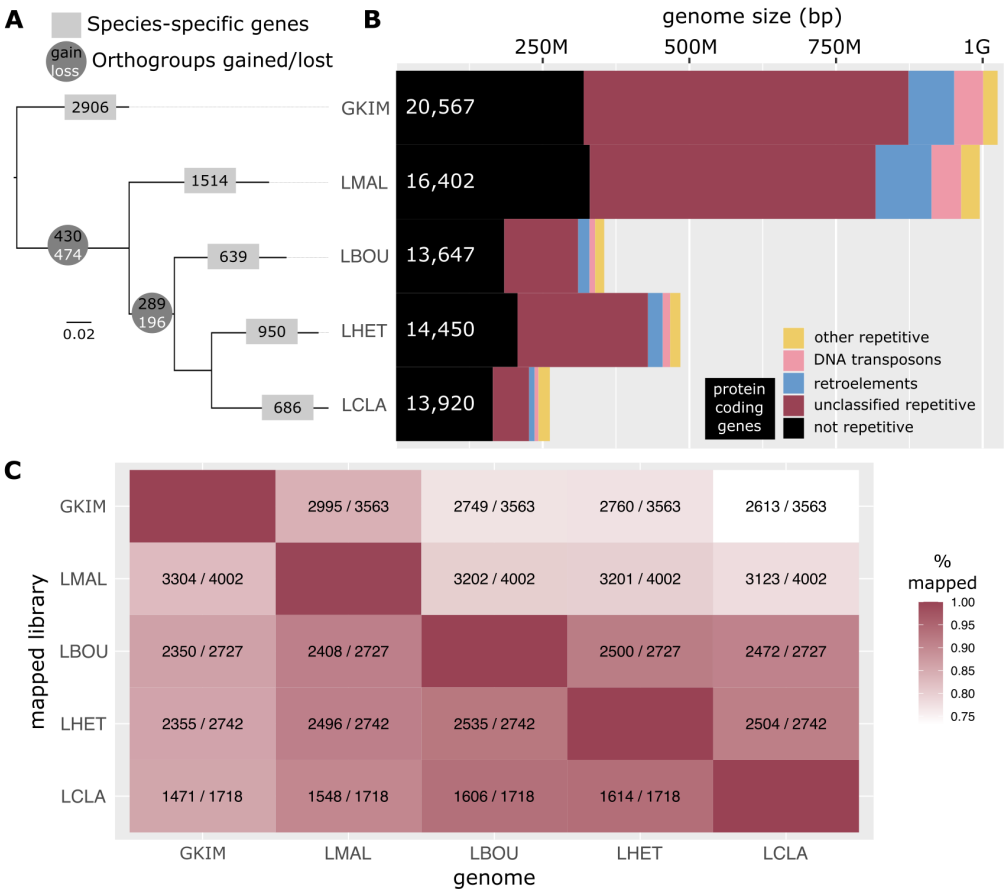
644 *Base pairs = bp
645
646 **Repetitive elements**
647 Given the larger than expected size of the *Leptopilina n. sp.* genome relative to other *Leptopilina* species,
648 we analyzed the repeat content of the assembly using a *de novo* repeat identification pipeline. 66.81% of
649 the nearly 1Gbp genome assembly was identified as repetitive (Figure 7, Supplementary Table S4).
650 Retroelements and DNA transposons only constituted 9.57% and 5.09% of the length of the assembly,
651 respectively. The majority of the repeat content (48.97% of the assembly) was categorized as unclassified
652 repetitive elements. To determine if this large repetitive fraction was unique to *Leptopilina n. sp.* we ran
653 the same *de novo* repeat identification pipeline on the three other *Leptopilina* species with genome

654 assemblies available, plus an outgroup from a closely related genus, *Ganaspis kimorum* (Supplemental
655 Table S4). Indeed, the other *Leptopilina* species were less repeat dense with 35.40%, 48.01%, and
656 57.27% repeat content for *L. clavipes, boulardi, and heterotoma,* respectively. Similar to *Leptopilina n.*
657 *sp.*, the majority of these repetitive elements were unclassified. In fact, the overall size and makeup of the
658 *Leptopilina n. sp.* genome was much more similar to the outgroup *Ganaspis kimorum* (Figure 7A,B).
659 However, the *Ganaspis kimorum* and *Leptopilina n. sp.* genomes contained approximately double the
660 amount of non-repetitive genome as compared to the other *Leptopilina* (black bars in Figure 7B). Thus,
661 while *Leptopilina n. sp.* had the largest repetitive content in the genus (both by total length and
662 percentage), repeat content alone does not completely explain the overall difference in genome size
663 relative to the other congenerics.
664
665 To understand the large differences in repeat content across the *Leptopilina* genomes, we performed an
666 all-versus-all analysis in which we mapped each species' repeat library to all other species' genomes
667 (Figure 7C). Then we determined how many of the unique repeat families were present to infer if there
668 was simply a difference in the number of repeat occurrences (e.g., family size), or, if entire repeat families
669 had been gained or lost across the clade. The most repeat dense genomes, *Ganaspis kimorum* and
670 *Leptopilina n. sp.*, contained 3,563 and 4,002 repeat families respectively, likely indicating a gain of
671 repeats in the *Leptopilina* common ancestor. Accordingly, while 84.1% of the *Ganaspis kimorum* repeat
672 families occur in the *Leptopilina n. sp.* genome, only 82.5% of the *Leptopilina n. sp.* repeat families occur
673 in *Ganaspis kimorum*. There appears to have been a large loss of repeat families in the ancestor of the
674 clade containing *L. boulardi*, *L. heterotoma*, and *L. clavipes*, and additional loss in the lineage leading to
675 *L. clavipes*. These genomes contained 2,727, 2,742, and 1,718 families respectively, and between 88-
676 91% of these families were found in *Leptopilina n. sp.*. Furthermore, while only 78-80% of the *Leptopilina*
677 *n. sp.* repeats were identified in these other *Leptopilina* genomes, an even lower percentage of the
678 *Ganaspis kimorum* repeats were present (73.3-77.5%). Finally, the comparisons within the clade of *L.*
679 *boulardi*, *L. heterotoma*, and *L. clavipes*, indicate high levels of shared repeats (85.8-93.9%). All together,
680 these data indicate that (1) the ancestor of the four *Leptopilina* species here acquired repetitive elements

681 that are not present in *Ganaspis kimorum*, (2) many of the repeats have since been lost in *L. boulardi*, *L.*

682 *heterotoma*, and especially in *L. clavipes*.

683




684

685 **Figure 7. Evolution of *Leptopilina* genomes. (A)** Species tree inferred by OrthoFinder, rooted on
686 outgroup, *Ganaspis kimorum*. Phylogeny is annotated with (1) the number of species-specific genes (gray
687 boxes), which include both singletons, and species-specific orthogroups, and (2) inferred gains and
688 losses of orthogroups. "Gained" orthogroups are those that are unique to and present in all taxa within the
689 clade, and "lost" orthogroups are present in all the outgroups, but none of the ingroups. **(B)** Genome
690 content of each representative assembly. **(C)** All-verus-all comparison of repetitive elements. Libraries of
691 the *de novo* identified repeats from each species were mapped to all other genomes, and the number of
692 unique repeat families recovered in each comparison is indicated within each box in the heat map
693 (number of families identified in "genome"/number of unique families in "library"). LMAL = *Leptopilina n.*
694 *sp.*

695

696 ***Wolbachia* Strain *w*Lmal**

697 We identified a circular *Wolbachia* genome in our assembly, hereinafter strain "*w*Lmal" (Table 4). A single

698 contig was sequenced at 344X coverage and blast results from blobtools revealed similarity to *Wolbachia*

699 strain *w*Ha from *Drosophila simulans* across most of the contig. BUSCO genome completeness metrics

700 were very high, with 99.2% of Rickettsiales BUSCOs present in single copy (Table 4). Phylogenetic

701 reconstruction based on core single-copy orthologs revealed that *w*Lmal is closely related to *Drosophila*
702 infecting *Wolbachia* strains (e.g., *w*Mel, *w*Ha*, w*Ri, *w*Au) in *Wolbachia* Supergroup A (Figure 8A). This is
703 notable not only due to the host-parasitoid relationship of *Leptopilina* and *Drosophila*, but also because
704 the parthenogenesis-inducing "*w*Lcla" strain from *Leptopilina clavipes* is quite divergent, and nested
705 within a different major *Wolbachia* clade: Supergroup B [59, 60]. The size (~1.2Mbp) and coding content
706 of the *w*Lmal genome is quite typical of an insect-infecting *Wolbachia* strain [61, 62] (Figure 8B, Table 4).
707 Two putative prophage regions were identified. The first (1,00,3471 bp - 1,051,238 bp) is partial and is
708 likely remnants of a *Wolbachia* "WO" prophage. This prophage region contains some phage structural
709 elements (primarily proteins for the tail) and what looks to be part of a "Eukaryotic Association Module"
710 (EAM), a region associated with WO phages which often contains proteins for manipulating host
711 reproduction and cell biology [63]. The second predicted prophage region (824,983 bp - 864,973 bp)
712 appears much more degenerate and only has a few remaining open reading frames encoding for phage
713 proteins.
714
715 We searched for the two recently identified putative parthenogenesis-inducing effector proteins, *pifA* and
716 *pifB,* which are found in the parthenogenesis inducing strains *w*Lcla and *w*Tpre [59]. *pifA* is also present
717 in the PI-*Wolbachia* infecting *Encarisa formosa* (designated *"piff"* in that strain) [64]. We identified two
718 identical copies of *pifB*, but did not find evidence for *pifA* in *w*Lmal (Supplemental File S1). Closer
719 inspection revealed that the *pifB* copies are in opposite orientations, and one copy of *pifB* is surrounded
720 by insertion elements and is just downstream of a putative *hin* DNA invertase, likely from the WO phage.
721 We also queried the *w*Lmal genome for a suite of other notable *Wolbachia* proteins and identified other
722 putative or confirmed effector proteins. Surprisingly, these included the genes *cifA* and *cifB* (here,
723 specifically "type I" or *cid*-type [65] which cause an entirely different host reproductive phenotype: the
724 conditional sperm-egg incompatibility known as cytoplasmic incompatibility (CI).
725
726
727
728

729 **Table 4. *Wolbachia* strain *w*Lmal genome assembly and annotation.**

| **Metric** | ***w*Lmal** |
|---|---|
| **Contigs** | 1 |
| **Length (bp)** | 1,198,858 |
| **%GC** | 35.1 |
| **BUSCO* (Rickettsiales)** | C:99.2%[S:99.2%,D:0.0%], F:0.3%,M:0.5%,n:364 |
| **CDS** | 1,356 |
| **rRNAs** | 3 |
| **tRNAs** | 34 |

730 *Standard BUSCO annotation: Complete BUSCOs (C) [Complete and single-copy BUSCOs (S),
731 Complete and duplicated BUSCOs (D)], Fragmented BUSCOs (F), Missing BUSCOs (M), Total BUSCO
732 groups searched (n)

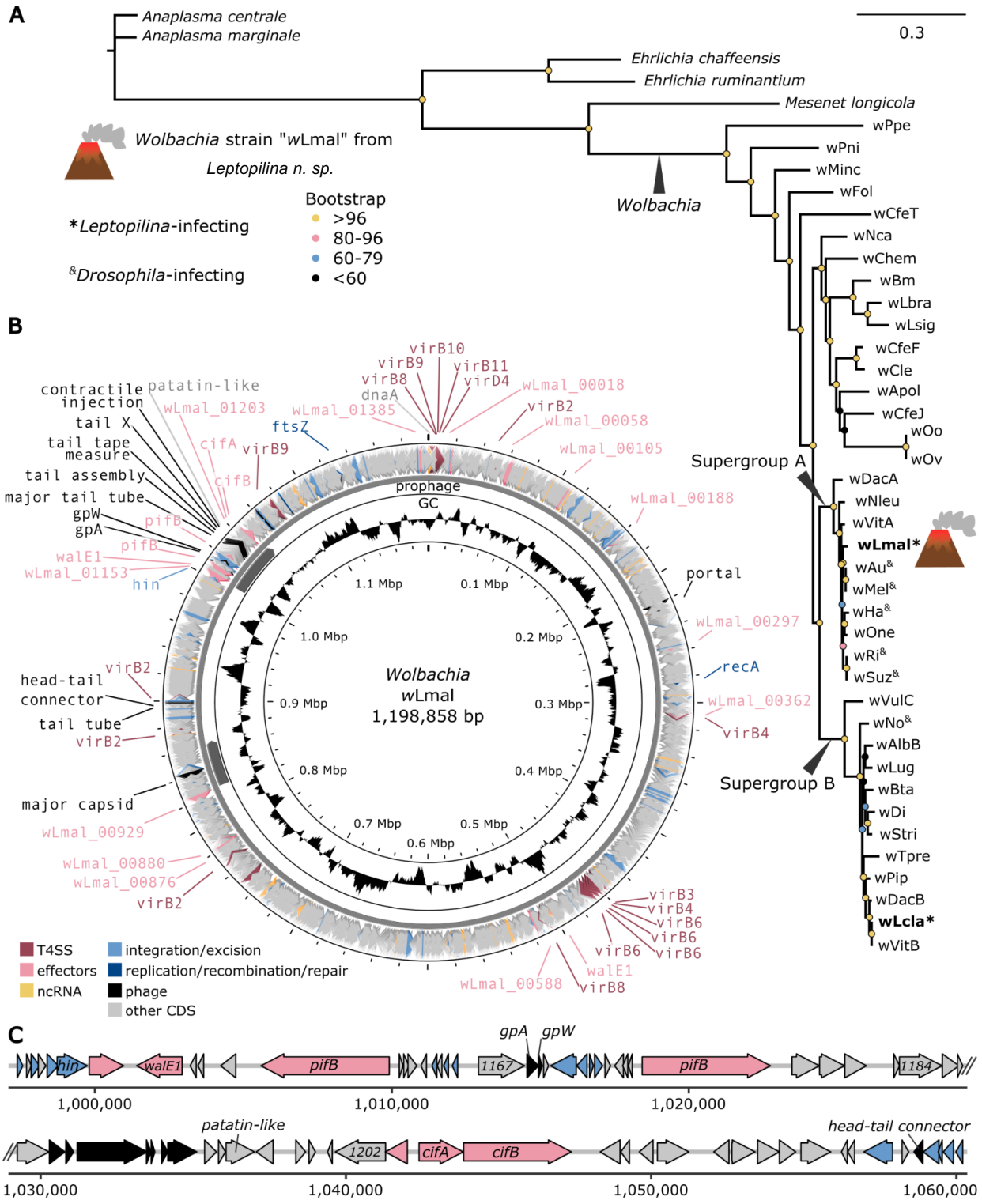



**Figure 8. *Wolbachia* strain *w*Lmal. (A)** Phylogenetic relationships of *Wolbachia* strains and Rickettsiales relatives based on 127 core single-copy orthologs. **(B)** Map of the complete *w*Lmal genome. Each concentric circle is annotated with a different set of genomic information. The innermost circle is annotated with a basepair marker, the second circle indicates GC content, the third circle indicates prophage regions identified by VirSorter, and the outermost circle shows the coding regions of the genome. Abbreviations: Type IV Secretion system (T4SS), noncoding RNA (ncRNA), coding sequences

740 (CDS). Putative effector proteins are indicated with the gene name where possible, otherwise the wLmal
741 CDS number is indicated and the *w*Mel homologs can be found in Supplemental Table S5. **(C)** Gene
742 models in the prophage region containing remnant WO phage structural proteins and a suite of effector
743 proteins homologs. Notably the two *pifB* coding sequences are present in an inverted repeat. Open
744 reading frames labeled with numbers (e.g., 1167) are gene annotation IDs, provided to benchmark the
745 region.
746

747 **Historical specimens of *Leptopilina n. sp.***

748 As noted above, our Saint Lucian specimens matched previously undescribed *Leptopilina* collected from
749 the Caribbean in the 1980's (specimens USNMENT01025775-USNMENT01025782). Specifically, those
750 *Leptopilina* (n=16, all female) were collected in 1982 and 1983 from Grande Terre, Guadeloupe (~270 km
751 from our collection site on Saint Lucia), some of which were used to initiate a now extinct colony, "PB10-
752 5". The PB10-5 wasps were previously characterized as an undescribed species nested within an early-
753 branching *Leptopilina* lineage, in the same clade as *Leptopilina longipes* [66, 67]. Our COI phylogenetic
754 reconstruction of *Leptopilina* recapitulated these relationships (Figure 6A). In addition to all the collected
755 Guadeloupe wasps being female, Yves Carton's notes indicated "*[the PB10-5 colony] emerged only*
756 *females: we can conclude that this species is parthenogenetic.*" In summary, our data indicate that
757 *Leptopilina n. sp.* was previously collected ~40 years ago on the neighboring island of Guadeloupe and
758 also reproduced via thelytokous parthenogenesis.
759

760 **DISCUSSION**

761 Our study represents a comprehensive description of *Leptopilina n. sp.*, the first recorded parasitoid of
762 *dunni* group *Drosophila* from their native range. By integrating classic taxonomy and systematics
763 approaches with genomics and experimental biology, we were able to generate a more comprehensive
764 view of the biology and evolution of this new species. Furthermore, historical records and museum
765 specimens enabled the discovery that asexually reproducing *Leptopilina n. sp.* have also been found on
766 another island in the Antilles. This work represents one of a limited number of studies in which description
767 of a new metazoan species is coupled with genome sequencing [68-72]. Indeed, this is the fifth such
768 example in insects [73-76], wherein body size often precludes whole genome sequencing of holotypes.
769

770 Despite the interest in speciation patterns of the *dunni* group *Drosophila*, the proximal ecological or
771 evolutionary mechanisms driving morphological and species diversity in this group remain unresolved.
772 One study found elevated nonsynonymous substitution rates in the pigmentation gene *yellow* in two
773 members of this group [77]. This is consistent with selection for pigmentation diversity, but no studies
774 have yet identified the selective agent responsible for this pattern. Importantly, melanization is a process
775 in many insect lineages that is critical in both the development of cuticle pigmentation as well as in
776 immune responses that occur during encapsulation of foreign pathogens [78]. In *Drosophila*, melanization
777 is a well-studied response to infection by eukaryotic parasites (including *Leptopilina*) and is linked to the
778 same genetic pathways as pigmentation production [79-81]. In one study in *Drosophila falleni*, selection
779 for a reduced number of abdominal spots resulted in higher infection rates by a parasitic nematode [82].
780 In other insects there are similar links between the levels or patterns of cuticular melanization and
781 resistance to parasites [83-88]. As such, it has been postulated that *Drosophila* abdominal coloration may
782 vary in some systems primarily due to selection by parasitism [89, 90], but direct evidence for this is
783 lacking.
784
785 Since adult flies of the *D. antillea* species are among the more highly pigmented flies of the *dunni* group,
786 we were interested in the possibility that they might be particularly resistant to infection by endemic
787 parasitoids. However, we instead found the opposite. *Leptopilina n. sp.* wasps are highly virulent on *D.*
788 *antillea* hosts. This observation could indicate that *Leptopilina n. sp.* is a relatively recent colonizer of
789 Saint Lucia and that *D. antillea* has not had sufficient time to evolve resistance. In contrast, *Leptopilina n.*
790 *sp.* was less effective at using *D. willistoni* as a host. Our findings open the door to future studies to
791 explore the potential mechanism of *Leptopilina n. sp.* virulence on flies of the *dunni* group as well as the
792 potential mechanisms of resistance for *D. willistoni.* It will also be of interest to test the virulence of
793 *Leptopilina n. sp.* on other species from the *dunni* group as well as how virulence tracks with island of
794 origin of the wasp. For example, are *Leptopilina n. sp.* wasps as effective when infecting *dunni* group flies
795 that are native to other islands of the Antilles chain? Are *Leptopilina n. sp.* wasps that originate from
796 Guadeloupe as virulent on *D. antillea*? Indeed, the island of Guadeloupe where Carton collected
797 *Leptopilina n. sp.* is home to the dunni group fly *Drosophila arawakana.* It would be of interest to know

798 how the virulence of Guadeloupian wasps relates to Saint Lucian wasps on fly species from the various
799 islands.
800
801 The island biogeography of *Leptopilina n. sp.* may also play a role in the evolution of their reproductive
802 mode. Our antibiotic curing experiments indicated that our *Leptopilina n. sp.* colony derived from Saint
803 Lucia is unable to revert to sexual reproduction. The inability to restore sex is a strong indicator of a so-
804 called "virginity mutation", which is well described in several other wasp species with symbiont-mediated
805 parthenogenesis [57, 91-96]. There are multiple hypotheses for the evolutionary pressures that lead to
806 virginity mutations [48, 91, 97, 98], but in brief, they are mutations that result in the decay or loss of
807 sexual function, to the point that the animal becomes reliant upon the PI symbiont for the production of
808 female offspring. Related to this, it is notable that the colony of *Leptopilina n. sp.* derived from
809 Guadeloupe collections in the 1980s were all female. However, we do not know if this PB10-5 colony was
810 (a) infected with *Wolbachia*, (b) asexual because of *Wolbachia*, or (c) obligately asexual (i.e., unable to
811 revert to a sexual mode). Indeed, some other populations of parasitic wasps with PI *Wolbachia* have
812 significant within-species polymorphism and vary in which *Wolbachia* strain they have, if they are
813 asexually reproducing, and if they have a so-called virginity mutation [48, 99]. Thus, it may be that
814 populations of *Leptopilina n. sp.* on different islands vary in whether they are sexually reproducing, and if
815 asexual reproduction is fixed or reversible. Notably, asexual reproduction across many taxa appears to be
816 more common on islands [100, 101], and thus *Leptopilina n. sp.* in the Greater Antilles may provide a
817 tractable system to understand the evolution of reproductive mode in the context of biogeography.
818
819 While there are many other examples of species with virginity mutations, our findings are quite unusual in
820 that the *Wolbachia* genome here also contains what appears to be functional genes for inducing
821 cytoplasmic incompatibility (CI). Paradoxically, the CI phenotype only manifests with sexual reproduction:
822 males with CI *Wolbachia*-modified sperm cause embryonic lethality in embryos derived from females
823 without a compatible *Wolbachia* strain [65]. We have several hypotheses for why these *Wolbachia*
824 encode for CI loci despite inducing parthenogenesis. First, the *cif* loci may have undergone some form of
825 neofunctionalization: perhaps these proteins are no longer involved in sperm-egg incompatibility as in CI

826 but are impacting other host processes. Alternatively, it might be that the transition to parthenogenesis is
827 more recent, and CI is no longer under selection. Perhaps the *cifs* are en route to be eliminated from the
828 genome in the future. “Recent” is of course difficult to determine quantitatively, especially in island
829 systems where there are often strong bottlenecks that can drive rapid evolution [102]. Finally, it might be
830 that if the PI phenotype has lower penetrance and occasional males are produced. These males might
831 mate with incompatible conspecifics (that either have no *Wolbachia* or an incompatible strain) and induce
832 CI, perhaps reducing competition for the asexual matriline. However, we see two issues with this
833 scenario. First, lower penetrance of PI phenotypes is typically due to the loss of *Wolbachia* (e.g., due to
834 higher temperatures, or in the lab, antibiotics), which leads to the production of males. In this case, the
835 occasional males produced by PI mothers might not have sufficient *Wolbachia* to induce CI. Second,
836 because the PI females transmitting *Wolbachia* to the next generation would never need to rescue CI,
837 there would be no selection pressure to maintain CI function.
838
839 Despite the peculiarity of a PI *Wolbachia* strain encoding for CI genes, all four loci associated with host
840 reproductive manipulation (*cifA, cifB,* and two copies of *pifB*) are found in the EAM of WO: a classic
841 signature of *Wolbachia* genomes and reproductive effector proteins. The multiple identical *pifB* copies,
842 genes for CI in combination with a clear *Wolbachia*-mediated PI phenotype, and evidence for multiple
843 prophage infections all point towards recent dynamism in the *w*Lmal genome. Many *Wolbachia* genes
844 mediating host reproductive switches are horizontally acquired from other *Wolbachia* strains, likely due to
845 the WO phage [63, 103]. For this reason, it was not surprising that the two PI *Wolbachia* strains from
846 *Leptopilina* both encoded for PifB proteins, despite the strains belonging to different clades. In contrast,
847 we did not find any evidence in *w*Lmal for the PifA protein, a *transformer* mimic that interacts with the
848 sexual differentiation pathway [59, 64]. Importantly however, we do not know the exact mechanism of
849 parthenogenesis induction employed by *w*Lmal (e.g., one versus two step, apomixis versus gamete
850 duplication, etc [104]). And, given the diversity of PI mechanisms found across *Wolbachia* [48] it is quite
851 possible that *w*Lcla and *w*Lmal are not using precisely the same toolset. Closer inspection of meiosis and
852 early embryonic mitoses in *Leptopilina n. sp.* will be key to understanding how *w*Lmal causes PI, and
853 what the role of PifB, or other proteins, might be in this process. Altogether, the description of *Leptopilina*

854 *n. sp.* and its reproductive biology will enable future explorations of host-parasitoid and host-symbiont
855 interactions, especially in the context of speciation.
856
857 In summary, this study provides a clear example of how descriptive work in under-studied species can
858 lead to novel discoveries across a broad set of biological disciplines. Insects generally, but especially
859 parasitic Hymenoptera, are under described [5]. Furthermore, of the described parasitic hymenopterans,
860 a significant majority lack biological information such as host associations and ecology. Here,
861 identification of a new species of parasitoid has provided a unique view into the intricate co-evolution of
862 host insect, parasitoid, and *Wolbachia* symbiont, with dynamic interactions between genomic evolution,
863 virulence, and reproductive ecology. We hope our work inspires more integration of descriptions of new
864 species coupled with investigations into novel aspects of their biology.
865
866 **DATA AVAILABILITY**
867 BioProject: PRJNA1150626
868
869 Supplemental File S1 contains:
870 Supplemental Text
871 Figure S1. Western Blot for *Wolbachia* detection.
872 Figure S2. Draft assembly snail plot and blob plot.
873 Supplemental Tables are provided in an excel file where each sheet corresponds to a different table
874 number:
875 Table S1. Passed reads statistics.
876 Table S2. All draft assembly contigs statistics, including BlobTools annotations.
877 Table S3. Purged contigs.
878 Table S4. Repeat annotations.
879 Table S5. *Wolbachia* protein queries.
880 Table S6. wLmal phage annotation.
881

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895

896 **CONFLICTS OF INTEREST**

897 MKD works for Oxford Nanopore Technologies.

898

899 **CITATIONS**

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