Supplementary information for

Two novel venom proteins underlie divergent parasitic strategies between a generalist and a specialist parasite

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Supplementary Figure 1 *Leptopilina heterotoma and L. boulardi* Representative images of *L. heterotoma* (Lh) adult male (a) and female (c), and *L. boulardi* (Lb) adult male (b) and female (d). Scale bar: 1 mm.



Supplementary Figure 2 Distribution of GC content across representative Parasitoida species.

RICB01, VOOK01, QYUC01, QYUB01, PHTE01, PQAT01, JUFY01 indicate NCBI accession numbers of other available *Leptopilina* genomes.



Supplementary Figure 3 Phylogenomics and orthology across representative hymenopteran species.

Orthology was analyzed across 10 representative hymenopteran species as follows. Gene repertoire of each species was divided to a given orthology type as indicated: "1:1:1", universal single-copy gene families across all examined species allowing absence or duplication in one genome; "N:N:N", other universal genes; "Parasitoida", orthologs specific to Parasitoida; "Leptopilina", orthologs specific to *Leptopilina*; "Patchy", all other orthologs across species; "S.D.", species-specific duplication; "Homology", genes with partial homology detected with $E < 10^{-5}$ but no orthology assigned; "N.D.", species-specific genes. The maximum-likelihood (ML) phylogenomic tree was calculated based on the concatenated alignments of 2704 exactly single-copy proteins in each species, rooted using *N. lecontei*. Bootstrap values are equal to 100 (out of 100 replicates) unless a number is labeled.



Supplementary Figure 4 Adaptive divergence between *L. heterotoma and L. boulardi* based on the nonsynonymous-to-synonymous substitution ratios (d_N/d_S) .

KEGG pathways to which all orthologous genes between *L. heterotoma* and *L. boulardi* were mapped are indicated by median dN/dS ratios and dN. Red dots indicate outlier pathways of potential signature of rapid evolution, while blue dots, as shown enlarged below, indicate pathways that are extremely conserved between Lh and Lb. See detailed information in Supplementary Data 1.



Supplementary Figure 5 Principle component analysis of Lh and Lb samples across different developmental stages and venom glands.

Analysis was based on overall expression of each sample. E, eggs; L1, days 1-3 larvae; L2, days 4-9 larvae for Lh while days 4-6 larvae for Lb; L3, days 7-9 larvae for Lb; P1, days 1-3 pupae; P2, days 4-7 pupae; P3, days 8-10 pupae; AF, female adults; AM, male adults; VG, venom glands. Names in red indicate Lh samples, while those in blue indicate Lb samples.



Leptopilina heterotoma

Leptopilina boulardi

Supplementary Figure 6 Expression heatmap of VG-highly expressed genes across developmental stages.

Expression is presented in log2 scale. Samples correspond to those in Supplementary Fig. 5.



Supplementary Figure 7 Sample distribution comparison between VG-highly expressed genes and all predicted genes.

Blue cells indicate expression in the corresponding developmental stage. Samples correspond to those in Supplementary Fig. 5.



Supplementary Figure 8 Heatmap of presented homolog of VG-highly expressed genes in other hymenopterans.

Blue cells indicate the presence of homology in the corresponding species. Lhet, *L. heterotoma*; Lbou, *L. boulardi*; Tpre, *T. pretiosum*; Nvit, *N. vitripennis*; Mdem, *M. demolitor*; Fari, *F. arisanus*; Hsal, *H. saltator*; Amel, *A. mellifera*; Pdom, *P. dominula*; Nlec, *N. lecontei*; Dmel, *D. melanogaster*.



Supplementary Figure 9 Origin distribution comparison between VG-highly expressed genes and all predicted genes.

Universal, genes presenting homology in at least eight out of the other nine hymenopteran species (see Supplementary Fig. 3 for species information); Patchy, genes presenting homology in several hymenopteran species; Leptopilina, genes presenting homologs only in Lh and Lb; -specific, species specific genes.

| а | | | | | | | | | | Exon-1 | << I >> | Exon-2 | | |
|--------------------------|----------------------------------------|----------------------------------------------------------------------|---------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------------------|-------------------------------------------|------------------------------------------------------|-------------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------------------|----------------------------------------------------------------------|-------------------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------|
| | 1 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 |
| Lar Lar' Consensus | GACAG | CAGGACGGT | GGCATTGAGT | TTAAAACAA A | AAGAATATTGA CGTGGAATTAA aagaaaATTaA | CATTCGTCT GTTTCGTCT CaTTCGTCT | ATTGTTCCT ATTGTTCCT ATTGTTCCT | GAATACCAAAT GAATATCAAAT GAATACCAAAT | ACAAGAAAA Acacgagaa Acaagaaaa | ATTTGACACCA AAGTTTCACAC AagTgaCACaa | AAACAGGTTT CAAGGACT aAA.,GGacT | TTAAATATCA TTAAAA TTAAAa | TATCAAC TATCAAC TATCAAC | TGGAAAA TG-AAAA TG.AAAA |
| | 131 | 140 | 150 | 160 | 170 | 180 | 190 | 200 | 210 | 220 | 230 | 240 | 250 | 260 |
| Lar Lar' Consensus | A <mark>A</mark> GTT ACGTT AaGTT | GTAATACCT GCACTACCT GCAaTACCT | TGTT <mark>GA</mark> TCA TGTTTGTCAA TGTT <mark>ga</mark> TCAA | ICATGATAA TCATGATAA TCATGATAA | GAATAGCAATA GAATAGCAATA GAATAGCAATA | ATCTGCTTT(ATCTGCTTT(ATCTGCTTT(| CTAGTTTCCA CTAGTTTCCA CTAGTTTCCA | TTGTTAGAAGT TTGT <mark>C</mark> AGAAGT TTGT <mark>C</mark> AGAAGT | CAGTTCTGT CAG <mark>GA</mark> CTGT CAG <mark>ga</mark> CTGT | AAGCTCACTTC GTGCTTAATTC aaGCTcAaTTC | TGATGATTTA TGATGAGTTA TGATGAgTTA | AATGGACTAA AATGAAGTAA AATGAACTAA | TCAATTGT GCAAATTT gCAAaTgT | TTAATGA TCAAGGA TcAAgGA |
| | 261 | 270 | 280 | 290 | 300 | 310 | 320 | 330 | 340 | 350 | 360 | 370 | 380 | 390 |
| Lar Lar' Consensus | TGTGC TGCGG TGCGC | ATAAAAGTG Ataaaagta Ataaaagta | TGGAGGAAGC TGGAAGAAGC TGGAaGAAGC | AACTCGAGG AACTAAAAA AACTaaAaa | TCAAAGTGGAA TCAAAGTGCAA TCAAAGTGCAA | ATGCGGCAA Atgcggcaa Atgcggcaa | ITTTTTTGGG ITGTCATAGG ITgTcaTaGG | CGACGTTGGAA CACCAGAAGAA CaaCagaaGAA | GTGAAAAAG GTGGAAAAA GTGaAAAAA | AAGCACTAATT GTACACTAATT aaaCACTAATT | AATTACTTAA AATTACTTGA AATTACTTGA | TAGGCAATGAI Teggcaatgai Taggcaatgai | ATTAATAGC ATTAACAGC ATTAACAGC | TT <mark>AC</mark> AGA TTTTAGA TT <mark>ac</mark> AGA |
| | 391 | 400 | 410 | 420 | 430 | 440 | 450 | 460 | 470 | 480 | 490 | 500 | 510 | 520 |
| Lar Lar' Consensus | CCAAA CGTGA CcaaA | TAAAT <mark>A</mark> TGA TAAAT T TGA TAAAT a TGA | AGCCCTAAAA Agacataaca AgacataAaa | CTGAGAAAA ATAAGAAAA aTaAGAAAA | GCTAACAATAG ACTAACGAAAA aCTAACaAaAa | TACCACAGG TACAACTGG TACAACAGG | CCAGAAATT CCAGAAATT CCAGAAATT | TATACTGGAAC AGTACTAGCGT aaTACTaGaac | AGTATCAAA AGAATTAGG AGaATcAaa | AACTAAAAATTO GTCTAAAAATTO aaCTAAAATTO | CTACAAAATG CTACAAAAATG CTACAAAAATG | G <mark>AA</mark> ATCAACAI GTCATCAAGAI GaaATCAACAI | 166 <mark>A</mark> CAGAA 166CCAGAA 166 <mark>a</mark> CAGAA | TTACAGA CTTCAAA cTaCAaA |
| | 521 | 530 | 540 | 550 | 560 | 570 | 580 | 590 | 600 | 610 | 620 | 630 | 640 | 650 |
| Lar Lar' Consensus | ATTTG Atgtg Atgtg | GAGATTTGG GACCTCTGG GAcatctGG | GATACTCCTC GATACTCCTT GATACTCCTC | CACTAATGG CAATTTTGG CAATaaTGG | ACAATAGAGGT ACAGCAGTGAT ACAacAGaGaT | CCACTGCAG CCAGTGCGT CCACTGCag | GATCTGAATA Gaattcagca Gaactcaaca | ATTCCATTTAT ATGCCCTTTAT ATgCCaTTTAT | TTGTATCAT CTGTATCAT CTGTATCAT | TTGATAAGAAG CTGATAAGAAG CTGATAAGAAG | TCTCGAATCA Agtcgaatct actcgaatca | CTAAAGTTTG CTGAAACTCA CTaAAacTca | FTGTAGTTA FTTTAGATG FTgTAGaTa | TCAATTG TCAATTT TCAATTg |
| | 651 | 660 | 670 | 680 | 690 | 700 | 710 | 720 | 730 | 740 | 750 | 760 | 770 | 780 1 |
| Lar Lar' Consensus | TCATG TAATT TaATg | ACATTATTT CCATTTTTT aCATTATTT | CTGATGATAT CTGATGATAT CTGATGATAT | TGGACAGAT TTATGAGAT TgaacAGAT | TCTCAGGCTTT TATTTTGCTTC TaTcagGCTTc | TACGTACTC TAAATGATC TAaaTaaTC | ITGAAACTTT ITGATCAATT ITGAaaaaTT | ATTTAATGACA ATTTAATGGCA ATTTAATGGCA | AGTTTAGAG TGTTTAAAG aGTTTAAAG | ATTTTTTGCCA ACTTTTTCCCT ActttttcCCa | AGTATATCAG AGTATATCAC AGTATATCAC | TTATTATTCI TAATTATCTCI TaATTATCTCI | AAGAGCACC AAAAGCACC AAAAGCACC | AGAAAAA TTATCAA agAaaAA |
| | 781 | 790 | 800 | 810 | 820 | 830 | 840 | 850 | 860 | 870 | 880 | 890 | 900 | 910 |
| Lar Lar' Consensus | ATTGA CACAA aacaA | AGATATTTT TGAAGCTAC aGAaacTac | AGTCGATCGT AGTTGATCAT AGTCGATCAT | GAATTTATA GAATATGTA GAATATATA | AAGAAAAAATT AGTGCCAAATT AagaaaAAATT | AGATTCGAA AAACTCGA- AaActCGA, | FATTATACAA FATTAGAAAG FATTAgAaAa | GATTCTACCTT TAATCTG gAaTCTa | AGTTATGTC -GTTGAGTC .GTTaaGTC | AAACGTTTCAA CCAAGTTTTAA aaAaGTTTCAA | GAGACTTTTT AAGACTTTAT AAGACTTTAT | ACGGCAGATTI ACAGCATATA ACaGCAgATal | ATGAGTAAT ACAGATAAT AcaaaTAAT | AAAAAAA AAAAAAA AAAAAAAA |
| | 911 | 920 | 930 | 940 | 950 | 960 | 970 | 980 | 990 | 1000 | 1010 | 1020 | 1030 | 1040 |
| Lar Lar' Consensus | GTGTA Gtgta Gtgta | GGCATCTTC GGCATCTTC GGCATCTTC | AGGAAACCGG AGGCTACCAA AGGaaACCaa | AAAATGTTG AAGATATTG AAaATaTTG | AAAAGGTTACT GAAA <mark>CC</mark> TTGCT aAAA <mark>CC</mark> TTaCT | GCTGTTATCO GCAGATATCO GCaGaTATCO | GATGATAATA Gatgataata Gatgataata Gatgataata | TTATTCAAGCT TTATTCCAGCT TTATTCAAGCT | ATTAATAAC Attaataac Attaataac | GCTGAG <mark>A</mark> GCAA GCTCAGGGCAA GCTCAG <mark>a</mark> GCAA | AGACAAGTCA Agacaag <mark>g</mark> ca Agacaag <mark>g</mark> ca | TCTAATCAGAI TCTCACCAGAI TCTaAcCAGAI | ATATAAGTT ATATGGGTA ATATaaGTa | TACCAAT TATCAAT TACCAAT |
| | 1041 | 1050 | 1060 | 1070 | 1080 | 1090 | 1100 | 1110 | 20 | 1130 | 1140 | 1150 | 1160 | 1170 |
| Lar Lar' Consensus | TTCAA TACAG TaCAa | TAGAATTGC AACGATATC aAcaATagC | ATCCTTGCCT TACCTTGCCT aaCCTTGCCT | GGAAGACAT GCAA <mark>C</mark> ACTT GCAA <mark>C</mark> ACaT | TAATTATTTGC TAATGATTTGA TAATgATTTGa | TGTCCAAAAA TGCACAAAAG TGcaCAAAAa | AGAAGCAAA ATAACCAAC AF AAcCAAa | AGAA GAA AGTG AGAAAAATAGTG AGAA <mark>a</mark> AaAGTG | CCTGATAAA TCTGATAAA CCTGATAAA | AATTTCTGGAA GATTTCTGGAG aATTTCTGGAa | TTTTATTTGC TTATTTATAC TTATATATAC | AGGCAGTA <mark>C</mark> T AGGCAGTA T T AGGCAGTA <mark>C</mark> T | ICTTCTAAT ICTTCTA-T ICTTCTA.T | TAAACTA AACTAAA aAaaaaA |
| | 1171 | 1180 | 1190 | 1200 | 1210 | 1220 | 1230 | 1240 | 1250 | 1260 | 1270 | 1281 | | |
| Lar Lar' Consensus | CTGTA CTACT CTaca | GTTATTATG GTTATTATG GTTATTATG | GGTTAATTCA GGTTAATTCA GGTTAATTCA | AATAATT AATAATTGT AATAATT | TTAGAAA AAACTTGAAAA TTaaAAA | ITATGGTGTC(ITATGGTGTC(ITATGGTGTC(| GGTTAATGTA GGTAACG GGTaAaa | TATCTAAATAT TTTCTACGTG- TaTCTAaaTa, | ARAAAATTA AAAATTA AAAATTA | АААААТАААТТ АААААТАААТТ АААААТАААТТ АААААТАААТТ | CCAGAATTTC CCAGAATTTC CCAGAATTTC | AAATTCT | | |
| b | 1 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 |
| Lar Lar Consensus | IMIRI IMIRI IMIRI | AIICFLVSI AIICFLVSI AIICFLVSI | VRSQFCKLTS VRSQDCVLNS VRSQdCkLnS | DDLNGLTQLF Delnevkqif D#lne1kqif | FNDVHKSVEEA FKDADKSHEEA FnDadKSnEEA | TRGQSGNAAJ TKNQSANAAJ TrnQSaNAAJ | FLGDVGSEK VIGTRRSGK fiGdrrSek | EALINYLIGNE STLINYLMGNE eaLINYLIGNE | LIAYRPNKY LIAFRRDKFI LIA%Rr#K% | EALKLRKANNS EDITIRKTNEN EaikiRKaN#n | TTGPEIYT <mark>GT</mark> TTGPEISTSV TTGPEIsTgt | VSKTKIPTKH ELGSKIPTKH elgskiptkh | STRTELQN SRRPELQN SrrpelQN | LETHDTP VDLHDTP 1#1HDTP |
| | 131 | 140 | 150 | 160 | 170 | 180 | 190 | 200 | 210 | 220 | 230 | 240 | 250 | 260 |
| Lar Lar Consensus | PLMDN SILDS pi\$Dn | RGPLODLNN SDPYREFSN rdP1r#1nN | SIYLYHLIRS ALYLYHLIRR aiYLYHLIRr | LESLKFYVVI VESLKLILD LESLK1!1d | ENCHDIISDDI /NFNSIFSDDI !NcndIiSDDI | GQILRLLRTL YEIILLLNDL g#IirLLrdL | ETLFNDKFR .DQLFNGMFK .#qLFNdkFr | DFLPSISVIIS DFFPSISLIIS DF1PSIS1IIS | RAPEKIEDI KAPYQHNEA rAPegh##a. | LYDREFIKKKL TYDHEYYSAKL LYDrE%!kaKL | DSNIIQDSTL NSILESNL #SIq#SnL | YHSNYSRDFL YESQYLKDFI YeS#Y1rDFir | ROIMSNKKS HITDNKKS gindnkks | VGIFRKP VGIFRLP VGIFRLP |
| | 261 | 270 | 280 | 290 | 300 | 310 | 321 | | | | | | | |
| Lar Lar Consensus | ENVEK KDIGN | TAVIDDNI AADIDDNI | IQAINNAESK IPAINNAQGK Igainna#ek | DKSSNQNISI DKASHQNHGI DKaSpQNigi | | EDINYLLSK | (EAKEESA | | | | | | | |

Supplementary Figure 10 Sequence alignment between Lar and Lar'.

a Alignment at the nucleotide level. Vertical line indicates the splicing boundary between the first and the second exon. The forward triangle indicates the translation start site; the downward triangle indicates the stop site of *Lar*, while the upward triangle indicates that of *Lar*'. **b** Alignment at the amino acid level. Alignment plots were generated using MultAlin^[1]. Note that the overall sequence identity of amino acids is 54%, which is even lower than that of nucleotides (73%), suggesting a signature of rapid evolution.

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Supplementary Figure 11 Putative modeled structures of *Lar* and its representative homologs.

a Alignment of Lar with the highest scoring template (c3zjcC, 20% identity and 99% confidence). For each set of alignment predicted by Phyre2^[2], rows from top to bottom indicate the predicted secondary structure of Lar, amino acid sequence of Lar, amino acid sequence of the template, the known secondary structure of the template, and the predicted secondary structure of the template, respectively. The 3D model structure is shown in **b**, along with modeled structures of its representative homologs: **c**, a copy of Sericostoma (group II); **d**, a copy of *N*. *vitripennis* (group I); **e**, a fungal copy of *Rhizophagus clarus* (outgroup).



Supplementary Figure 12 Lar is specifically expressed in the VG of *L. heterotoma*.

a Immunolocalization of Lar (green) in Lh venom gland (n=3 replicates, at least 20 lymph glands were examined for each individual). **a'** Merged image of Lar staining (green) and nuclei stained with DAPI (blue). Scale bars: 20 μ m. **b** Western blot analysis of Lar in parasitoid venom apparatus and carcass (n=3 replicates). CBB: Coomassie Brilliant Blue. The same amount of proteins was loaded after quantification with BCA Protein Assay kit, and CBB is the indicator of loading samples.



Supplementary Figure 13 Parasitic efficacy assays for different dsRNA-treated Lh. a Relative mRNA levels of *Lar*, *Lar*' and *LhOGS20047* in Lh after RNAi treatments. Three biological replicates were performed. Data are means±SD; Significance was determined by two-tailed unpaired Student's t-test (*dsLar*': P=0.0026; *dsLar*: P=0.0001; *dsLhOGS20047*: P=7.7e-5). b Percentage of host larvae exhibiting lytic lymph gland 24 h after parasitization

by dsGFP-treated Lh (P_{dsGFP}, n=123), dsLar'-treated Lh (P_{dsLar'}, n=116), dsLar-treated Lh (P_{dsLar}, n=120), and dsLhOGS20047-treated Lh (P_{dsLhOGS20047}, n=127). Three biological replicates were performed. Data are means±SD; Significance was determined by two-tailed unpaired Student's t-test (P_{dsLar}: P=0.8527; P_{dsLar}: P=1.8e-5; P_{dsLhOGS20047}: P=0.011). c Percentage of host larvae containing encapsulation capsules after parasitization by the above dsRNA-treated Lh wasps (P_{dsGFP}, n=893; P_{dsLar}, n=951; P_{dsLar}, n=693; P_{dsLhOGS20047}, n=1082). Three biological replicates were performed. Data are means±SD; Significance was determined by two-tailed unpaired Student's t-test (P_{dsLar}: P=0.0134; P_{dsLar}: P=4.1e-5; P_{ds1h0GS20047}: P=0.0016). **d** Parasitism rate in host larvae after parasitization by dsRNA-treated wasps in c. Three biological replicates were performed. Data are means±SD; Significance was determined by two-tailed unpaired Student's t-test (P_{dsLar}: P=0.3505; P_{dsLar}: P=0005; $P_{ds1h0GS20047}$: P=0.0809). e Wasp emergence rate in host larvae after parasitization by dsRNA-treated wasps in c. Three biological replicates were performed. Data are means±SD; Significance was determined by two-tailed unpaired Student's t-test ($P_{dsLar'}$: P=0.639; P_{dsLar} : P=0008; $P_{dsLhOGS20047}$: P=0.318). *:P < 0.05; ***: P < 0.005; ****: P < 0.001; ns: not significant.



Supplementary Figure 14 Lar contributes to suppress the lamellocyte differentiation and encapsulation immune responses.

a Lamellocytes, labeled by the Msn>mCherry marker (Red) were rarely found in host post-infection by dsGFP-treated Lh (P_{dsGFP}). However, lamellocytes were largely induced upon dsLar-treated Lh infection (P_{dsLar}) (n=3 replicates, at least 50 *Drosophila* larvae were examined for each individual). Scale bars: 1 mm. **b** There was no encapsulation responses due to the lack of lamellocytes in P_{dsGFP} infected host larvae. Then, the wasp egg successfully developed to larval stage 48 h later. The triggered lamellocytes initiated to adhere to the wasp egg at 24 h, encapsulated the wasp egg at 36 h, and completely encapsulated and melanized the wasp egg at 48 h in host larvae after infection by P_{dsLar} (n=3 replicates, at least 50 wasps were examined for each individual). Scale bars: 20 µm.



Supplementary Figure 15 The distribution of Lar in different host tissues.

Fluorescent images of fat body, gut and salivary gland from unparasitized host (non-P) and parasitized host (P_{Lh}). Tissues were dissected 24 h after parasitization by *L*. *heterotoma* females, and stained with anti-Lar (red) and DAPI (blue) (n=3 replicates, at least 20 *Drosophila* tissues were examined for each individual). Scale bars: 50 µm.



Supplementary Figure 16 RNAi and parasitic efficacy assays for other candidate venom proteins of high expression.

a Relative mRNA levels of *LhOGS06609*, *LhOGS10118*, *LhOGS01638*, *LhOGS01639*, *LhOGS01180*, *LhOGS02019*, *LhOGS00546*, *LhOGS20077*, and *LhOGS08557* in Lh after RNAi treatments. Three biological replicates were performed. Data are means ± SD; Significance was determined by two-tailed unpaired Student's t-test (*dsLhOGS06609*: *P*=6.2e-6; *dsLhOGS10118*: *P*=6.0e-6; *dsLhOGS01638*: *P*=0.0001; *dsLhOGS01639*: *P*=2.9e-5; *dsLhOGS01180*: *P*=7.7e-7; *dsLhOGS02019*: *P*=6.9e-5; *dsLhOGS00546*: *P*=0.0002; *dsLhOGS20077*: *P*=0.0007; *dsLhOGS08557*: *P*=0.0001). **b** Parasitism rate in host larvae after

parasitization by dsRNA-treated wasps in a. Three biological replicates were performed. Data are means \pm SD; Significance was determined by two-tailed unpaired Student's t-test ($P_{dsLhOGS06609}$: P=0.0388; $P_{dsLhOGS10118}$: P=0.4854; $P_{dsLhOGS01638}$: P=0.604; $P_{dsLhOGS01639}$: P=0.633; $P_{dsLhOGS01180}$: P=0.1825; $P_{dsLhOGS02019}$: P=0.256; $P_{dsLhOGS00546}$: P=0.3684; $P_{dsLhOGS20077}$: P=0.7134; $P_{dsLhOGS08557}$: P=0.0651). **c** Percentage of host larvae exhibiting lytic lymph gland 24 h after parasitization by dsGFP-treated Lh (P_{dsGFP} , n=123), dsLhOGS06609-treated Lh ($P_{dsLhOGS06609}$, n=83). Three biological replicates were performed. Data are means \pm SD; Significance was determined by two-tailed unpaired Student's t-test ($P_{dsLhOGS06609}$: P=0.2782). *:P < 0.05; ****: P < 0.001; ns: not significant.



Supplementary Figure 17 Gene arrangement around the *Lar* locus across three *Leptopilina* species.

Orthologous genes were shown in the same color. Note that the first intron of HELQ (shown in the dashed box) is presented in another scaffold of the Lc genome. Numbers shown below the scale line (white) indicate genomic coordinates in Kb. *Lar* was reversely inserted into the first intron of *RRP8*, whose detailed information is shown below across three species. Note that the length of box is scaled to the proportion to the total length but the actual length. Red boxes indicate *Lar*, while others correspond to those noted on the right.



Supplementary Figure 18 Local synteny around the *Lar* locus across three *Leptopilina* species.

a Genome alignment between the genomes of Lh and Lc; **b** that between Lh and Lb; **c** that between Lc and Lb. **d** Phylogeny relationship among the three species. Shadows in red indicate the locus of Lar.



Supplementary Figure 19 Local synteny around the *Lar'* locus across three *Leptopilina* species and *N. vitripennis*.

Orthologous genes were shown in the same color. Unfilled exons in the same color indicate absence in the Lb genome. The grey dashed box outlined in places of Lh indicates that exons were massively lost in the first 42-Kb of Lbou_1230 and the last 4-Kb of Lbou_4801, which is homologous to the location of *Lar*' in Lh. The large region between serotriflin and *Lar*' is full of repeats. Note that some regions without any genic features were scaled up to fit the overall presentation; see numbers labeled below, indicating genomic coordinates in Kb, for detailed information.



Supplementary Figure 20 Multiple alignment of all further homologs of *Lar* in the Lh and Lb genomes.

Only the 170-aa conserved region is shown. The four G motifs (in green) are referred to the previous study^[3]. However, only G1-motif is conserved across homologs, as shown in a green box, and agrees with the previous prediction (GxxxGKS/T), while the other three G motifs are all poorly conserved and do not agree with the previous prediction^[3] (G2: T; G3: DxxG; G4: T/SKVP).



Supplementary Figure 21 Multiple alignment of representative homologs of *Lar.* **a** Multiple alignment of *Lar* homologs in Chalcidoidea. **b** Multiple alignment of representative *Lar* homologs across different subgroups. The phylogenetic trees were independently calculated based on the multiple alignment using MEGA7^[4] under the JTT model. G-motif sites correspond to those in Supplementary Fig. 20. Species initiated with I, II, III, and OM indicate copies from groups I, II, and III and outgroup as shown in Fig. 3. Species in shadow indicate copies of *N. vitripennis*. Note that *N. vitripennis* is the only species that simultaneously encodes copies of clades I and III.





Each dot indicates the genetic distance (*K* distance) between two homologs from the respective group. All homologs were subdivided into two groups based on the expression level in VG. As control, a total of 2492 co-lineage orthologous gene pairs between Lh and Lb were identified.



Supplementary Figure 23 Distribution of taxa documented with IPR004954 in the InterPro database.

a The pie chart showing proportion of accumulated species within each genus being documented with IPR004954. Genus names in red indicate considerable presence in the microbiota sequencing of Lb, while those in blue indicate a little presence (Supplementary Data 9). **b** The pie chart showing proportion of accumulated sequences within each genus being documented with IPR004954.



Supplementary Figure 24 RNAi efficacy for 9 mucin-binding domain-containing genes in Lb.

Relative mRNA levels of *LbOGS00358*, *LbOGS02280*, *LbOGS02281*, *LbOGS04370*, *LbOGS05722*, *LbOGS06929* (*Warm*), *LbOGS06930*, *LbOGS08145* and *LbOGS09927* in *L*. *boulardi* after RNAi treatments. Three biological replicates were performed. Data are means \pm SD; Significance was determined by two-tailed unpaired Student's t-test (*dsLbOGS00358*: *P*=7.9e-6; *dsLbOGS02280*: *P*=7.5e-5; *dsLbOGS02281*: *P*=0.0048; *dsLbOGS04370*: *P*=0.0001; *dsLbOGS05722*: *P*=0.003; *dsLbOGS06929*: *P*=0.0007; *dsLbOGS06930*: *P*=8.1e-5; *dsLbOGS08145*: *P*=0.0002; *dsLbOGS0927*: *P*=3.3e-5). ***: *P* < 0.005; ****: *P* < 0.001.



Supplementary Figure 25 Different developmental stages of *Leptopilina* species for transcriptome analysis.

Eight samples of Lh and nine samples of Lb were collected for the transcriptome sequence. E, eggs; L1, days 1-3 larvae; L2, days 4-9 larvae for Lh while days 4-6 larvae for Lb; L3, days 7-9 larvae for Lb; P1, days 1-3 pupae; P2, days 4-7 pupae; P3, days 8-10 pupae; AF, female adults; AM, male adults.



Supplementary Figure 26 K-mer distribution across the Lh and Lb genomes. a the 17-mer distribution across the Lh genome. **b** the 17-mer distribution across the Lb genome. The plot was generated using GenomeScope (github.com/schatzlab/genomescope).

| Sequencing mode | Insert size | Read length (N50) | Mean read length | #Reads | Total length (Gb) | Coverage (x) |
|---------------------|----------------|-------------------------|------------------------|--------------|-------------------------|-----------------|
| Pacbio Sequel | 20 Kb | 15,813 | 9,144 | 3,395,220 | 31.05 | 63.8 |
| Illumina Paired-end | | 150 | 150 | 80,048,286×2 | 24.01 | 49.3 |

Supplementary Table 1 Sequencing statistics of the Lh genome

| | Leptopilina heterotoma | Leptopilina boulardi |
|-------------------------------|------------------------|----------------------|
| Genome assemble | | |
| Assembly size (bp) | 487,015,184 | 323,668,388 |
| #Scaffolds | 411 | 9,872 |
| Scaffold N ₅₀ (bp) | 2,183,205 | 458,625 |
| Contig size (bp) | 487,015,184 | 299,861,858 |
| #Contigs | 411 | 38,756 |
| Contig N ₅₀ (kp) | 2,183,205 | 14,385 |
| Gene annotation | | |
| Protein-coding | 11,881 | 11,054 |
| Genomic features | | |
| GC (%) | 26.94 | 25.77 |
| Coding (%) | 4.9 | 8.8 |
| Quality control | | |
| BUSCO partial (%) | 98.7 | 97.2 |
| BUSCO complete (%) | 98.5 | 95.1 |
| CEGMA partial (%) | 98.4 | 97.6 |
| CEGMA complete (%) | 90.7 | 94.4 |

Supplementary Table 2 Basic features of the assembled genomes of Lh and Lb

| | | | | ~ Benome | |
|--------------|-------------|--------|-------------|----------|----------|
| | | Read | | Total | Covoraço |
| Library mode | Insert size | length | #Read pairs | length | (v) |
| | | (bp) | | (Gb) | (X) |
| Paired-end | 180 bp | 125 | 25,564,639 | 6.39 | 21.3 |
| Paired-end | 300 bp | 125 | 37,145,732 | 9.29 | 31.0 |
| Paired-end | 450 bp | 250 | 22,375,641 | 11.19 | 37.3 |
| Mate-pair | 2 Kb | 125 | 26,047,574 | 6.51 | 21.7 |
| Mate-pair | 3 Kb | 125 | 13,822,559 | 3.46 | 11.5 |
| Mate-pair | 5 Kb | 125 | 14,178,383 | 3.54 | 11.8 |
| Mate-pair | 8 Kb | 125 | 12,480,118 | 3.12 | 10.4 |
| Mate-pair | 13 Kb | 125 | 30,459,170 | 7.61 | 25.4 |

Supplementary Table 3 Sequencing statistics of the Lb genome

| Species | Leptopilina | heterotoma | Leptopilina | boulardi |
|-------------------|-------------|------------|-------------|----------|
| Class | Length (bp) | % Genome | Length (bp) | % Genome |
| Retroelements | 23,063,394 | 4.74% | 11,006,358 | 3.40% |
| SINEs: | 2,064,383 | 0.42% | 284,727 | 0.09% |
| Penelope | 520,623 | 0.11% | 173,681 | 0.05% |
| LINEs: | 11,050,478 | 2.27% | 4,910,145 | 1.52% |
| L2/CR1/Rex | 587,532 | 0.12% | 533,853 | 0.16% |
| R1/LOA/Jockey | 2,916,696 | 0.60% | 559,000 | 0.17% |
| R2/R4/NeSL | 40,952 | 0.01% | 26,216 | 0.01% |
| RTE/Bov-B | 50,075 | 0.01% | 16,120 | 0.00% |
| L1/CIN4 | 15,487 | 0.00% | 7,606 | 0.00% |
| LINE1 | 0 | 0.00% | 58,153 | 0.02% |
| LINE2 | 1,214,680 | 0.25% | 201,338 | 0.06% |
| L3/CR1 | 253,782 | 0.05% | 1,921,501 | 0.59% |
| LTR elements: | 9,948,533 | 2.04% | 5,811,486 | 1.80% |
| BEL/Pao | 1,099,859 | 0.23% | 728,983 | 0.23% |
| ERVL | 15,284 | 0.00% | 0 | 0.00% |
| ERV_classI | 19,889 | 0.00% | 0 | 0.00% |
| ERV_classII | 46,697 | 0.01% | 0 | 0.00% |
| Ty1/Copia | 1,069,378 | 0.22% | 791,645 | 0.24% |
| Gypsy/DIRS1 | 4,952,234 | 1.02% | 2,649,768 | 0.82% |
| DNA transposons | 37,457,347 | 7.69% | 15,184,067 | 4.69% |
| Hat-Charlie | 37,481 | 0.01% | 293,139 | 0.09% |
| TcMar-Tigger | 32,129 | 0.01% | 35,390 | 0.01% |
| hobo-Activator | 169,427 | 0.03% | 80,763 | 0.02% |
| Tc1-IS630-Pogo | 165,140 | 0.03% | 113,204 | 0.03% |
| PiggyBac | 8,634 | 0.00% | 11,204 | 0.00% |
| Tourist/Harbinger | 15,502 | 0.00% | 19,605 | 0.01% |
| Other | 42,426 | 0.01% | 30,013 | 0.01% |
| Unclassified: | 190,157,436 | 39.05% | 83,172,397 | 25.70% |
| Total | 250,678,177 | 51.47% | 109,362,822 | 33.79% |

Supplementary Table 4 Statistics of repeat content in the Lh and Lb genomes

| | Lh | Lb |
|---------------------------------------|--------|--------|
| Approach for gene prediction | | |
| Maker (Consensus) #genes | 11,864 | 11,013 |
| Braker #genes | 38,798 | 22,202 |
| StringTie #genes | 13,641 | 13,645 |
| ToFU #genes | 7246 | 9815 |
| OGS (further filtered based on Maker) | 11,881 | 11,054 |
| Mean exon per gene | 6.2 | 6.6 |
| Median exon per gene | 5 | 5 |
| Mean exon length (bp) | 289 | 352 |
| Mean intron length (bp) | 2356 | 1841 |
| Genes w/ annotations | | |
| Transcriptome evidence | 11,079 | 10,574 |
| Hymenopteran homology | 11,259 | 10,087 |
| Drosophila homology | 8973 | 8094 |
| KEGG KO | 7697 | 6891 |
| Gene Ontology | 6626 | 5910 |
| InterPro domain | 9473 | 8472 |
| Pfam domain | 8884 | 7947 |
| NCBI RefSeq (invertebrate) | 11,323 | 10,158 |
| UniProt (Hymenoptera) | 11,271 | 10,079 |

Supplementary Table 5 Official gene sets of Lh and Lb

| Samula ID | Somela information | RNAseq data | #Expressed | #Highly |
|-----------|---------------------|-------------|------------|-----------------|
| Sample ID | Sample mormation | (Gb) | genes | expressed genes |
| Lh | | | | |
| Е | Egg | 7 | 6245 | 143 |
| L1 | Larva (day 1-3) | 6.8 | 6485 | 138 |
| L2 | Larva (day 4-9) | 6.4 | 5795 | 98 |
| P1 | Pupa (day 1-3) | 7 | 6602 | 69 |
| P2 | Pupa (day 4-7) | 6.7 | 6375 | 94 |
| P3 | Pupa (day 8-10) | 6.8 | 7028 | 138 |
| AF | Female adult | 7.3 | 6113 | 170 |
| AM | Male adult | 6.9 | 6819 | 71 |
| VG | Venom glands | 7.3 | 4274 | 39 |
| | Pooled ^a | 8.6 | 6184 | - |
| Lb | | | | |
| Egg | Egg | 7.4 | 5511 | 103 |
| L1 | Larva (day 1-3) | 7.7 | 6374 | 126 |
| L2 | Larva (day 4-6) | 7.4 | 4975 | 102 |
| L3 | Larva (day 7-9) | 8.6 | 4593 | 92 |
| P1 | Pupa (day 1-3) | 9.1 | 6287 | 111 |
| P2 | Pupa (day 4-7) | 7 | 6372 | 108 |
| P3 | Pupa (day 8-10) | 6.9 | 5898 | 79 |
| AF | Female adult | 7.2 | 5602 | 128 |
| AM | Male adult | 7.2 | 6229 | 84 |
| VG | Venom glands | 7.3 | 4538 | 28 |
| | Pooled ^a | 22 | 8459 | - |

Supplementary Table 6 Statistics of RNAseq data in this study

^aThis sample was pooled from independent samples above equally and subject for full-length RNAseq using the PacBio platform. We mainly used for gene prediction but expression quantification.

| | | | | Best | Best | |
|------------|-------|-------|-------|----------------|---------------|-------------------|
| | E- | Query | | BLASTP hit | BLASTP hit | IPR004954 |
| Gene ID | value | cover | Score | (Accession ID) | genus | (InterProScan) |
| LbOGS02281 | 4e-20 | 86% | 107 | WP_142464257 | Klebsiella | 504-613(1.5e-9) |
| LbOGS04370 | 3e-16 | 80% | 95.5 | WP_142466376 | Klebsiella | 551-660 (6.7e-11) |
| LbOGS09927 | 6e-7 | 38% | 66.2 | WP_079940407 | Paenibacillus | 542-650 (4.2e-8) |
| | | | | | | 670-776 (1.9e-6) |
| | | | | | | 798-908 (1.9e-8) |
| LbOGS06929 | 1e-5 | 13% | 60.8 | WP_050103721 | Yersinia | 861-970 (1.6e-9) |
| LbOGS02280 | 0.003 | 16% | 53.5 | WP_039946932 | Anaerostipes | 693-802 (7.8e-10) |
| LbOGS05722 | 0.017 | 57% | 46.2 | TCW59216 | Bacillus | 57-165 (3.5e-11) |
| LbOGS00358 | 0.051 | 31% | 45.4 | VEA39472 | Salmonella | 176-286 (7.4e-10) |
| LbOGS06930 | 0.13 | 35% | 48.9 | WP_051492772 | Listeria | 717-824 (3e-7) |
| | | | | | | 868-970 (5.8e-7) |
| LbOGS08145 | 0.1 | 10% | 48.9 | ECQ2803623 | Salmonella | 542-650 (9.4e-8) |
| | | | | | | 670-776 (1.9e-6) |
| | | | | | | 798-906 (1.1e-8) |

Supplementary Table 7 BLASTP of Warm homologs

Supplementary References

[1] Corpet, F. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* **16**, 10881-10890 (1988).

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