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Analysis of insecticide resistance and *de novo* transcriptome assembly of resistance associated genes in the European grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae)

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Abstract

The European grapevine moth *Lobesia botrana* (Denis & Shiffermüller 1776) is an economically important pest of the vine-growing areas worldwide. Chemical insecticides have been used for its control; however, its resistance status is largely unknown in many regions. We monitored the susceptibility of several *L. botrana* populations from Greece and Turkey. In addition, based on RNAseq transcriptome analysis, we identified and phylogenetically classify the cytochrome P450 genes of *L. botrana*, as well as analysed target site sequences and looked for the presence of known resistance mutations. Resistance against chlorantraniliprole, alpha-cypermethrin, spinetoram, etofenprox, and acetamiprid was very low (below 2.5-fold in all cases, compared to a reference strain from Greece) in all populations from Greece that were included in the study. However, resistance against indoxacarb (4–30-fold), spinosad (5–59-fold), and deltamethrin (18–30 fold) was detected in the *L. botrana* populations from Turkey, compared to a reference population from Turkey. De novo transcriptome assembly and manual annotation, and subsequent PCR-based analysis of insecticide target sequences (i.e. voltage-gated sodium channel – VGSC: target of pyrethroids and oxadiazines; nicotinic acetylcholine receptor subunit a6 – nAChR_α6: target of spinosad; ryanodine receptor – RyR: target of diamides; glutamate-gated chloride channel – GluCl: target of avermectins and; acetylcholinesterase – AChE: target of organophosphates) showed the absence of known resistance mutations in all specimens from both countries. Finally, the *L. botrana* CYPome (116 genes) was manually analysed and phylogenetically characterised, to provide resources for future studies that will aim the analysis of metabolic resistance.

Introduction

Lobesia botrana (Denis and Shiffermüller, 1776), commonly known as the European grapevine moth is an economically important pest of the vine-growing areas of North Africa, several countries of Asia and Europe as well as North and South America (Lucchi and Scaramozzino, 2022). *L. botrana* feeds predominantly on grapes but it also has a host range across almost 27 plant families, a characteristic that contributes to the establishment of the pest in a wide range of ecological zones (Altimira *et al*., 2021). The larvae feed on flower clusters and berries which can subsequently facilitate the development of harmful fungi such as *Botrytis cinerea* and *Aspergillus* (Cozzi *et al*., 2013; Delbac and Thiéry, 2016).

Mating disruption using synthetic sex pheromones, natural enemies, biopesticides, and sterile insect techniques have been applied against *L. botrana* (reviewed in Benelli *et al*., 2023). However, chemical control, using insecticides has been the most common practice in many geographical regions, such as the Mediterranean Basin, including Greece and Turkey. Several insecticides have been used for *L. botrana* in these countries, including neonicotinoids (acetamiprid) avermectins (abamectin and emamectin benzoate), pyrethroids (acrinathrin, cypermethrin, deltamethrin, etofenprox, lambda-cyhalothrin and tau-fluvalinate), spinosyns

(spinosad and spinetoram), diamides (chlorantraniliprole), oxidiazines (indoxacarb) and *Bacillus thuringiensis* (subsp. Aizawai and Kurstaki).

Despite a number of control failure reports, there are only a few confirmed resistance cases in *L. botrana*. For example, indoxacarb resistance has been identified through field trials and laboratory bioassays in Emilia-Romagna (Italy) and Manisa (Turkey) (Civolani *et al.*, 2014; Durmuşoğlu *et al.*, 2015; Hatipoğlu *et al.*, 2015).

Insecticide resistance mechanisms primarily include modification of target sites and/or enhancement of detoxification (ffrench-Constant, 2013). Cytochrome P450s (P450s), glutathione S-transferases, and carboxylesterase are the key metabolic detoxification gene families (Li *et al.*, 2007; Pavlidi *et al.*, 2018; Nauen *et al.*, 2022). Target site resistance, conferred by alterations (typically point mutations) on the molecular target of the insecticide, has been reported in several gene/subunit targets, including: nicotinic acetylcholine receptors against neonicotinoids and spinosyns (Silva *et al.*, 2016); acetylcholinesterases against organophosphates (Feyereisen *et al.*, 2015); voltage gated sodium channels (knock-down resistance) against pyrethroids; glutamate gated chloride channels against avermectins (Casida and Durkin, 2013; ffrench-Constant, 2013) and ryanodine receptor against diamides (Douris *et al.*, 2017).

Monitoring insecticide resistance, using bioassays and/or molecular diagnostic tools (Van Leeuwen *et al.*, 2020) is important to design and apply evidence-based insecticide resistance management (IRM) strategies. However, the analysis of insecticide resistance mechanisms at the molecular level and the identification of specific molecular markers for resistance have been hampered by the lack of genomic information in *L. botrana*.

The aim of this study was to monitor the susceptibility of *L. botrana* populations from Greece and Turkey, against different insecticides registered and used for their control. Molecular analysis of the insecticide target site was also performed, aiming to identify known conserved resistance mutations. Finally, the *L. botrana* CYPome was identified and phylogenetically characterised.

Materials and methods

Insect collection and rearing

Seven *L. botrana* populations from Crete, Greece were collected from the following locations: Vagiona, Episkopi, Archanes, Alagni, and Ano Archanes (table S1). The laboratory susceptible reference strain (LB-S), which originated from Bordeaux France, and maintained under laboratory conditions for more than 20 years was used as a reference strain, for the analysis of resistance ratio factors in this subgroup (*L. botrana* populations from Greece).

Five populations of *L. botrana* were collected from different locations in Turkey, respectively: Manisa-Merkez, Manisa-Alaşehir, Manisa-Ahmetli, Manisa-Saruhanlı, Denizli. One population (namely S strain), was collected for Kahramanmaraş area, more than 700 km away from Manisa and Denizli areas (fig. 1) where viticulture is less intense and pest control is practically absent. This field population (Kahramanmaraş) was used as a control, to calculate resistance levels, for the populations collected in Turkey.

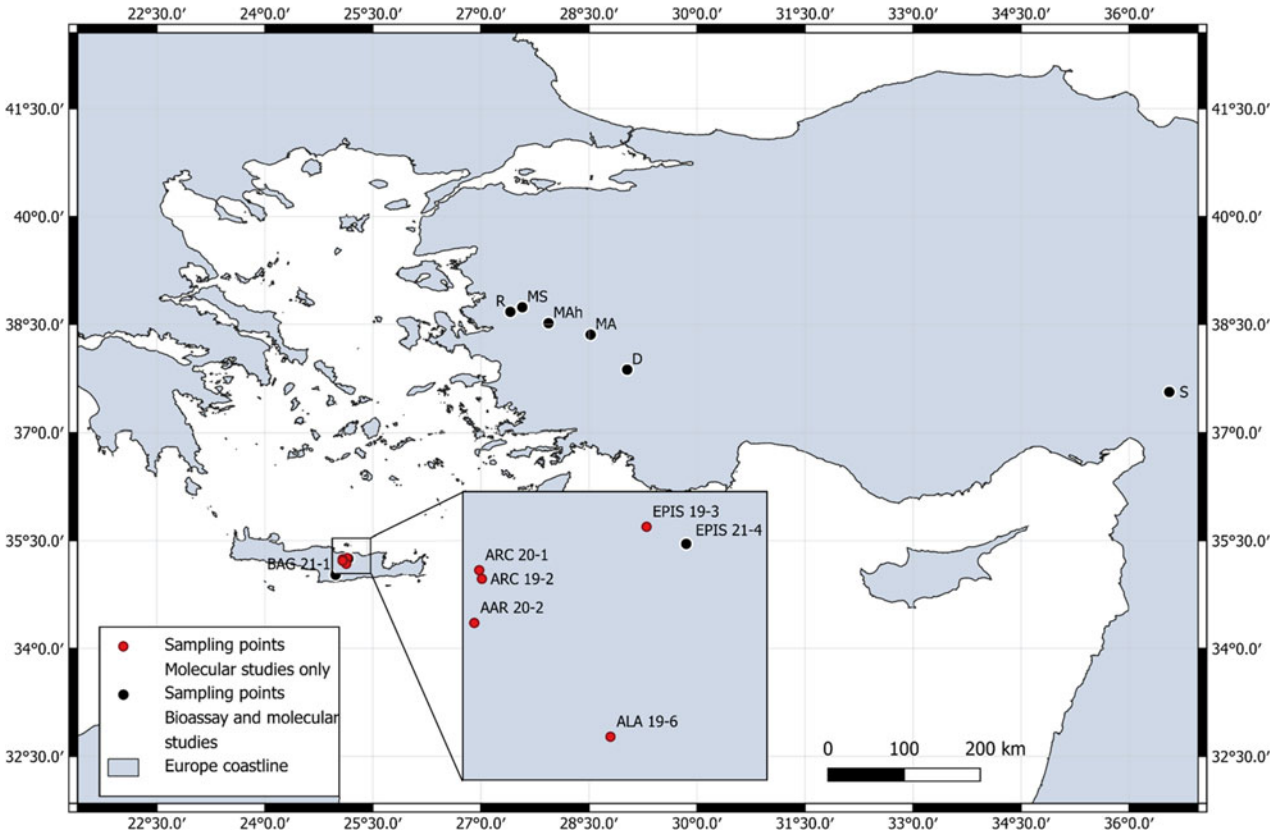

Figure 1. The collection sites for the *L. botrana* populations tested, were in Greece and Turkey. S: Kahramanmaraş strain (susceptible reference strain), R; Manisa-Merkez strain, MA: Manisa-Alaşehir strain, MAh: Manisa-Ahmetli strain, MS: Manisa-Saruhanlı strain, D: Denizli strain.

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The exact location of all the collection sites is shown in fig. 1, while a detailed record for each population from both Greece and Turkey is provided in table S1. For each population, an assigned code name was used. Insects were collected as larvae of different stages from infested grape berries. The collected larvae were transferred into trays with artificial diet, where they started feeding and completed their life cycle. Emerging adults were used to establish respective strains from each population under laboratory conditions. The insect-rearing protocol for the Greek and the Turkish populations was according to Mironidis and Savopoulou-Soultani, 2008 and Durmuşoğlu *et al.*, 2015, respectively. All populations were maintained at 25 ± 2 °C and 60–65% relative humidity in the laboratory with a fixed 16:8 (L:D) photoperiod. The Greek strains were maintained on an artificial diet for two to three generations.

Insecticides

The insecticides tested in bioassays *L. botrana* populations from Greece were the following: the pyrethroids (group 3A-IRAC classification) etofenprox (Therbonal 28.75 EC, Mitsui Chemicals, Tokyo) and alpha-cypermethrin (Fastac 10 EC, BASF S.E., Germany); the neonicotinoid (group 4A) acetamiprid (Carnadine 20 SL, Nufarm, Australia); the spinosyns (group 5) spinetoram (Radiant 120 SC, Corteva, Switzerland) and spinosad (Laser 480 SC, Dow, USA); the avermectin (group 6) emamectin benzoate (Affirm 095 SG, Syngenta, UK); the oxadiazine indoxacarb (Stewart 30 WG, DuPont, France) and; the diamide (group 28) chlorantraniliprole (Coragen 20 SG, DuPont, Switzerland).

The insecticides tested in bioassays with *L. botrana* populations from Turkey were the following: the pyrethroid (group 3A) deltamethrin (Decis 25 EC, Bayer, Germany); the oxadiazine (group 22A) indoxacarb (Avaunt® 150 EC, DuPont, France); the spinosyn (group 5) spinosad (Laser 480 SC, Dow, USA).

Bioassays

The methodology used for analysing the susceptibility to insecticides of *L. botrana* populations from Greece was a slightly modified version of IRAC method 017. The modified method IRAC017 was initially validated against the standard IRAC017, by conducting dose response bioassays, on the susceptible reference strain LB-S.

Briefly, for the standard IRAC017 method, insecticide dilutions were mixed with ready to use stone fly diet mix (growth substrate previously tested for supporting *L. botrana* development (data not shown) (38–0600 Stonefly Heliothis Diet) at a ratio of 1 solution:40 diet, based on producers instructions. Small aliquots of the insecticide/diet mix were placed into individual cells of a 16-well polystyrene bioassay tray (BioServe, USA). A single neonate (1st instar larvae <24 h old) was placed into each well and the tray was covered and left for 96 h in the same conditions described previously for insect rearing (25 ± 2 °C, 60–65% RH and 16:8 h L:D). For the preparation of the modified IRAC017 method, while the final diet mixture was still in liquid form (at 40 °C), insecticide dilutions were added at a ratio of 1: 40. The mixture was allowed to cool down for 30 min. Once the diet became solid it was divided in bioassay trays. In each tray, 10 neonates were placed and left for 96 h as previously described. For both bioassay protocols (standard and modified IRAC017) all insecticide concentrations were calculated at the final diet volume. The mortality was assessed after 96 h (4 days) by counting dead and moribund larvae which were unable to make coordinated movement when they were gently poked with a fine brush.

A two-step approach was implemented in the resistance studies for Greek populations, using the modified IRAC017 protocol. First, there was implemented a single-dose bioassays approach, for putative resistance detection, followed by dose–response experiments for accurate resistance levels estimation. Briefly, full-dose experiments on the susceptible reference strain allowed the determination of the LC_{95} for each insecticide tested. This value was used as a diagnostic dose for putative insecticide resistance detection on the wild strains. The % mortality was assessed, using the aforementioned bioassay protocol. If the observed mortality at the diagnostic dose did not exceed 80%, the tested strains were considered as candidates for resistance development and full dose–response experiments were designed to estimate the extract LC values and the potential resistance levels. For each dose–response experiment 5–6 sequential insecticide concentrations were used resulting in mortality levels ranging between 0 and 100%. For each dose, three replicates were performed, with a total of 20 insects per dose. Insecticide concentrations were calculated as mg per L of diet and ranged between 0.01 and 1.0 for chlorantraniliprole, 30–300 for acetamiprid, 0.15–30.0 for alpha-cypermethrin, 43–1438 for etofenprox, 0.03–0.30 for spinetoram, 0.16–0.48 for spinosad, 0.01–0.48 for emamectin benzoate, and 1.5–4.5 for indoxacarb. All insecticide concentrations were predefined by preliminary range finder tests. In some cases, the total number of insects per bioassay was marginally lower than the minimum of 120, but some biological limitations posed by *L. botrana* (i.e. number of neonates available in a single day) made it very difficult to achieve.

Here it should be mentioned that the Greek and Turkish *L. botrana* populations were handled separately, using different methodologies. The bioassays for the *L. botrana* populations collected from Turkey were conducted using the diet incorporation method described by Durmuşoğlu *et al.*, 2015. The insecticides were mixed with the artificial diet (mixture at 40 °C) while for control treatment, sterile distilled water was used at a ratio of 1: 9. The mixture was homogenised, left for 24 h at room temperature and then separated in 1 cm^3 cubes. The cubes were placed into individual cells of the 16-well polystyrene bioassay tray to conduct the bioassays.

A single 3rd stage larva was placed into each well and the tray was covered and maintained at 25 ± 2 °C, 60–65% RH and a 16:8 h light:dark photoperiod. For the bioassay, six insecticide concentrations and a control were used. Each concentration included twenty 3rd stage larvae. Insecticide concentrations ranged between 0.02 and 200 mg l^{-1} for deltamethrin and spinosad, while for Indoxacarb the concentration ranged between 0.05 and 20 mg l^{-1}. The mortality was assessed after 72 h, and larvae were considered dead if they were unresponsive to gentle prodding with a fine brush. If a larva failed to grow further to the pupal stage, it was recorded as dead. Each bioassay was repeated twice.

Statistical analysis

Mortality data from dose–response bioassays were subjected to probit analysis based on Finney (1964) using PriProbit 3.4 (Sakuma, 1998) or Polo Plus (LeOra Software Inc., Berkeley, CA, USA). Both types of software test the linearity of dose–mortality response and provide the slope, the lethal concentrations (LC), and the 95% confidence limits (CL) of the lethal concentration for each mortality line. Using the appropriate function, the relative potency ratio among responses was calculated. Responses were considered significantly different when the 95% confidence interval of relative potency ratio did not include the

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value 1. Percentage mortality values generated in bioassays were corrected using Abbott's formula (Abbott, 1925). Resistance ratios (RR) were calculated by dividing the LC_{50} value of the resistant strains by that of the susceptible strain.

RNA extraction and cDNA synthesis

For the transcriptome analysis, L. *botrana* larvae of the Kahramanmaraş population (S) and the Manisa-Merkez (R) populations from Turkey were placed in microcentrifuge tubes and rapidly frozen in liquid nitrogen before storage at −80 °C. Total RNA was extracted from of mixed-age insects using a total RNA purification kit (GeneMark, Taiwan) according to the manufacturer's instructions. DNase treatment followed, to eliminate the presence of gDNA in the RNA samples. Agarose gel electrophoresis and spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, USA) were used to assess the integrity and the concentration of each RNA sample respectively. RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

For the target sequencing analysis, total RNA was extracted from all seven populations from Crete (table S1), originating from pools of 20 L2 instar larvae/population using Trizol reagent (MRC, Cincinnati, OH, USA), according to the manufacturer's instructions (1 ml of TRIzol per prep). RNA samples were treated with Turbo DNase (Ambion, Foster City, CA, USA) to remove genomic DNA. Then, 3 µg of the treated RNA was used to generate first-strand cDNA using oligo-dT20 primers with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Library preparation and transcriptome sequencing

A total amount of 3 µg RNA per sample was used as input material for RNA library preparations. Sequencing libraries were generated using NEB Next® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEB Next First Strand Synthesis Reaction Buffer (5x). First-strand cDNA was synthesised using random hexamer primer and M-MuLV Reverse Transcriptase (Rnase H-). Second-strand cDNA synthesis was subsequently conducted using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEB Next Adaptor with a hairpin loop of 150–200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index Primer. At last, PCR products were purified (AMPure XP system) and the library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 150 bp paired-end reads were generated. The sequencing reads are available from the Sequence Read Archive (SRA) under the BioProject accession PRJNA827155.

De novo transcriptome assembly

Reads from both the resistant and the susceptible population were first quality-trimmed with Trimmomatic v0.39 (Bolger *et al.*, 2014) with default parameters. The trimmed reads were then assembled de novo using Trinity v2.5.1 (Grabherr *et al.*, 2011), with parameters '-seqType fq –max_memory 50G'. The assembled transcriptome is available from the Transcriptome Shotgun Assembly (TSA) under the BioProject accession PRJNA827155.

Analysis of loci containing conserved target site insecticide resistance mutations

The raw reads were then mapped onto the unfiltered Trinity contigs using Hisat2 (Kim *et al.*, 2019) and the generated SAM files were converted to sorted BAM files using SAMtools (Li *et al.*, 2009). VarScan v2.4.4 (Koboldt *et al.*, 2012) was used to detect statistically significant SNPs in genes that are known targets of specific insecticides (table S3). The *Lobesia* target genes were searched in the de novo assembled transcriptome using BLAST. More specifically, the amino acid sequence of the genes that are known to be involved in insecticide resistance in other species was BLASTed against the *Lobesia* transcriptome. Finally, the SNPs were manually inspected in the Integrative Genomics Viewer v2.6.3 (Robinson *et al.*, 2011).

Analysis of loci containing conserved target site insecticide resistance mutations by PCR and sequencing

cDNA was used as a template for PCR amplification of target-site gene fragments encompassing insecticide resistance mutations, identified in various arthropod species and highly linked with insecticide resistance (table S3). For the ace gene of *L. botrana* specific primers were designed based on the available mRNA on NCBI (accession number: JQ771363.1). For the rest of the genes of interest, primers were designed in conserved gene regions (table S3) based on available mRNA sequences of ten Lepidopteran species in NCBI. PCR reactions (50 µl) contained 1ul cDNA, 0.4 mM primers, 0.2 mM dNTPs, 5 µl of 10 × buffer, and 1U Kapa Taq DNA polymerase (KAPABIOSYSTEMS). The thermal conditions were: 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 54–60 °C for 30 s (depending on the primer set for the gene of interest in table S3), 72 °C for 60 s, and final extension at 72 °C for 2 min. PCR products were purified using NucleoSpin Extract II (Macherey-Nagel) and sequenced directly on PCR products with the original PCR primers (table S3). Sequencing reactions were performed at GENEWIZ (Germany). Sequencing data were analyzed with BioEdit v7.2 (Hall, 1999). The presence/absence of target-site mutations was also based on visual examination of sequence chromatographs.

Identification of cytochrome P450 (CYP) genes

To identify the coding sequences of genes in the predicted Trinity transcripts and obtain the encoded proteins, TransDecoder programme (included in the Trinity suite) was used with the default parameters. From the resulting set of predicted protein contaminants, originating from ingested plant material were removed. To

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achieve this filtering step, a Diamond (Buchfink *et al.*, 2015) search of all the Trinity proteins against the Uniref50 (Suzek *et al.*, 2015) database was used. Using a set of custom Perl scripts we filtered out proteins whose first hit was bacterial, plant, or viral. Also using custom Perl scripts, we kept only the longest isoform per gene, by using the Trinity naming scheme and finally removed any identical sequences using CD-HIT (Fu *et al.*, 2012), with parameters '-n 5 -c 1.00 -M 64000'. Completeness was assessed using BUSCO v3.0.2 (Waterhouse *et al.*, 2018). This set of unigenes is available as a fasta file in the Supplementary Material (Supplementary File S1). InterProScan v5.28–67 (Jones *et al.*, 2014) was subsequently run on the final set of unigenes and the ones containing the IPR001128 InterPro domain were considered to be CYPs. In addition, we searched the entire set of unigenes using BLAST (Camacho *et al.*, 2009), against a curated collection of 2942 CYPs from a wide variety of arthropods (Dermauw *et al.*, 2020). *L. botrana* unigenes with significant similarity (*e*-value <1*e*-05), to any of the curated CYPs were also considered to be candidate CYPs. Unigenes identified by either method (InterProScan or BLAST hit against the curated CYPs) constituted our final set of *L. botrana* CYPs. The set of all *L. botrana* P450s is also available as a fasta file in the Supplementary Material (Supplementary File S2).

A phylogenetic analysis was conducted using the herein identified *L. botrana* CYPs, using the curated CYPome of the related cotton bollworm *Helicoverpa armigera* (Dermauw *et al.*, 2020) as a reference. More specifically, the amino acid sequence of the CYPomes of the two species were aligned using MAFFT (Katoh and Standley, 2013) with the 'auto' parameter. Next, the alignment was trimmed using Trimal (Capella-Gutierrez *et al.*, 2009), with the 'automated1' parameter. RaxML (Stamatakis, 2014) was used for reconstructing a maximum likelihood phylogeny with 100 bootstrap replicates. Moreover, the human CYP51A1 was used as an outgroup and the automatic selection of the amino acid model ('PROTGAMMAAUTO'). Finally, Evolview v2 (He *et al.*, 2016) was used for drawing and decorating the phylogenetic tree.

Results

Bioassay data – Lobesia botrana populations from Greece

The LC_{50} provided by the modified IRAC017 bioassay protocol were compared to the LC_{50} of the standard IRAC017 protocol for the insecticides chlorantraniliprole, alpha- cypermethrin, spinetoram, spinosad, etofenprox and acetamiprid (table S2). No statistically significant differences were detected in the response of the strain when using either bioassay approach (table S2). In addition, the chi-square values were either identical or lower in the modified IRAC017 bioassay protocol suggesting higher accuracy and reliability of the results, associated with the proposed method modification. This protocol was implemented in the toxicological studies hereafter.

Single-dose bioassays were conducted on wild strains using the LC_{95} of the susceptible strain (LB-S) as diagnostic dose. As shown in table S2, the diagnostic dose was 151 mg l^{-1} for etofenprox, 1.02 mg l^{-1} for alpha-cypermethrin, 36.1 mg l^{-1} for acetamiprid, 0.12 mg l^{-1} for spinetoram, 0.47 mg l^{-1} for spinosad, 0.2 mg l^{-1} for emamectin benzoate, 3.37 mg l^{-1} for indoxacarb and 0.39 mg l^{-1} for chlorantraniliprole. The percentage mortality rates for the insecticides emamectin benzoate, spinosad, and indoxacarb exceeded 80% at the diagnostic dose, (94.4–100% mortality in all wild strains), therefore full dose-response bioassays were not conducted for these chemicals. Chlorantraniliprole, acetamiprid, alpha-cypermethrin, etofenprox and spinetoram exhibited mortality levels below 80% at the diagnostic dose. To accurately evaluate the suspected resistance levels, full dose-response experiments were implemented. The responses of the wild populations were compared against the susceptible reference strain and the results are shown in table 1. In all cases, control mortality was found below 5% and the responses of the populations to the insecticides were homogenous and fitted the log-dose probit-mortality model. The LC_{50} for the insecticide chlorantraniliprole was estimated between 0.13 and 0.18, for alpha-cypermethrin between 0.92 and 1.58, for spinetoram between 0.06 and 0.12, for etofenprox between 90 and 102 and for acetamiprid at 43 (all values in mg l^{-1}). The resistance levels (RR) of the wild populations from Greece to all insecticides were below 2-fold with the exception of alpha-cypermethrin where resistance scaled up to 3- and 5-fold (table 1).

Bioassay data – Lobesia botrana populations from Turkey

In all the *L. botrana* populations from Turkey (mentioned in table 2), full dose–response bioassays for the insecticides deltamethrin, spinosad, and indoxacarb were performed (table 2). The population Kahramanmaras LC_{50} numbers for deltamethrin, spinosad, and indoxacarb were 1.22, 0.27, and 0.35 mg l^{-1}, respectively.

The LC_{50} values of the tested populations varied from 21.66–36.57, 1.44–15.64, and 1.37–10.34 mg l^{-1} for deltamethrin, spinosad, and indoxacarb, respectively. The respected resistance ratio was up to 30-fold for deltamethrin and indoxacarb and up to 59-fold for spinosad. The Manisa-Merkez (R strain) exhibited the highest LC_{50} values in all three tested insecticides in all three tested insecticides (table 2).

Transcriptome assembly

A *de novo* transcriptome was assembled for *L. botrana* using the Trinity programme with the two Illumina libraries that were sequenced in the frame of this study. A total of 169,945 transcripts were assembled that are grouped into 98,064 unigenes (table S4). This transcriptome contains the complete sequence of 84% of the Insecta BUSCO, thus being fairly complete. A total of 43,857 proteins were predicted from the assembled transcripts. This set of proteins was first filtered in order to exclude contaminants, such as sequences with similarity to plant proteins (n = 43,322 proteins survived). Subsequently, only one protein per unigene was kept (n = 22,847 proteins survived) and as a last filtering step proteins identical with other proteins in the unigene set were excluded (n = 22,803 proteins survived). The BUSCO pipeline found the complete sequence for 79.5% of the Insecta data set. Such a considerable decrease (by 4.5%) compared to the unfiltered protein set is most probably due to the draft nature of gene prediction that is implemented here.

Analysis of insecticide targets for the presence of resistance mutations

Based on the *de novo* assembled *L. botrana* transcriptome (for resistant and susceptible populations from Turkey), as well as subsequent PCR and sequence investigations (populations from Greece), the presence of known resistance mutations or putative novel polymorphism in the insecticide binding sites associated with insecticide resistance was investigated (table S3 and fig. 2).

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Table 1. Log dose probit mortality data for *L. botrana* populations from Greece

Strain	*N*	LC_{50}	CL 95%	RR		LC_{90}	CL 95%	Slope	s.e.	χ^2	df	*p*
Chlorantraniliprole												
LB-S	93	0.09	0.05–0.14		*a*	0.28	0.18–1.07	2.53	0.73	1.91	2	0.38
BAG 21–1	112	0.13	0.002–0.28	1	*a*	3.57	1.12–748,640	0.88	0.38	3.88	2	0.14
EPIS 21-4	91	0.18	0.002–0.42	2	*a*	2.22	0.83–16,244	1.20	0.51	0.61	1	0.44
Alpha-cypermethrin												
LB-S	99	0.35	0.22–0.39		*a*	0.78	0.56–1.61	3.14	0.72	2.71	2	0.26
BAG 21-1	97	0.92	0.48–1.35	3	*b*	2.16	1.45–7.16	3.46	0.32	0.17	2	0.92
EPIS 21-4	108	1.58	0.9–2.29	5	*b*	4.03	2.70–10.9	3.15	0.87	0.09	2	0.96
Spinetoram												
LB-S	108	0.06	0.04–0.07		*a*	0.10	0.08–0.16	5.68	1.48	0.09	2	0.96
BAG 21-1	160	0.12	0.1–0.15	2	*a*	0.49	0.20–0.42	4.27	0.95	2.35	4	0.67
EPIS 21-4	108	0.06	0.04–0.07	1	*a*	0.13	0.09–0.21	3.78	0.74	2.28	2	0.32
Etofenprox												
LB-S	89	77.7	49.88–96.76		*a*	130	103–288	5.70	1.91	0.23	1	0.63
BAG 21-1	102	56.8	27.62–85.88	1	*a*	207	131–577	2.28	0.58	1.55	2	0.46
EPIS 21-4	90	132	74.64–192.75	2	*a*	373	246–1030	2.85	0.77	0.55	2	0.76
Acetamiprid												
LB-S	119	24.1	19.11–27.96		*a*	33.1	28.3–58.6	9.34	3.17	1.47	2	0.48
BAG 21-1	108	42.6	25.67–53.76	2	*a*	117	83.6–367	2.91	0.87	0.54	2	0.76
EPIS 21-4	93	43.2	20.55–63.11	2	*a*	120	81.6–267	2.89	0.77	1.34	2	0.51

LB-S, laboratory susceptible reference strain; BAG 21-1, Bagiona strain; EPIS 21-4, Episkopi strain; *N*, number of larvae tested; CL, confidence limits; RR, resistance ratio; LC50 in mg l^{-1}, χ^2 testing linearity of dose–mortality response: resistance ratio (RR) is based on strain LB-S.
*Different letters indicate significant differences in the responses ($P < 0.05$).

The conditions and primer sets described above and in table S3 respectively, were used for this analysis, which included the insecticide targets VGSC (pyrethroids and oxadiazines); nAChR_α6; spinosad; RyR (diamides); GluCl (avermectins) and; AChE (organophosphates). The analysis revealed the absence of resistance mutation in all resistant or suspected resistance populations included in this analysis (nine in total: seven from Greece and two from Turkey, as mentioned in subsection RNA extraction and cDNA synthesis.).

The CYPome of lobesia botrana: annotation and phylogenetic characterisation

We identified 161 CYPs in the *L. botrana* transcriptome assembly (table S5). Only 49 of these CYPs appear to be full-length (>450 amino acids) with the remaining 112 unigenes being apparently fragmented. This fragmentation is probably due to the low RNA sequencing depth. Despite the occurrence of many fragmented CYPs, three of the four insect P450 clans (2, 3, and Mito) are well-supported with bootstrap values >65% (fig. 3). Additionally, many conserved families and subfamilies within each clan are also well-supported. The CYP4 clan, however, has a bootstrap support of 39%, which is considered to be relatively low.

Discussion

Low to moderate insecticide resistance levels indoxacarb (4–30 fold), spinosad (5–59 fold), and deltamethrin (18–30 fold) were detected in *L. botrana* populations from Turkey, compared to a susceptible population from the same geographical region. However, resistance against chlorantraniliprole, alpha-cypermethrin, spinetoram, etofenprox, and acetamiprid was not detected (below 2.5 fold in all cases) in *L. botrana* populations from Greece, indicating that the phenomenon is not responsible for control failures often reported in the region. Other parameters, than insecticide efficacy, could be associated with these failures, such as the accurate estimation of application time (based on pest flight density), which is critical for the efficiency of the application (Benelli *et al.*, 2023). Cultural practices and appropriate spray coverage of the grape bunches may also affect the efficacy of an application at a technical level.

The absence of high-level resistance to insecticides (i.e. compared to other lepidopteran species) in *L. botrana* populations from Turkey and Greece, despite the history of intensive insecticide application in both countries for many years might be partially attributed to the few generations of *L. botrana* (3 generations) per year (Siddiqui *et al.*, 2023). The ability of *L. botrana* to develop some generations on alternative host plants (more than 40 wild and cultivated plants i.e. *Olea europea*, *Drimia maritima*) (Ioriatti *et al.*, 2011) that do not receive insecticide application in combination with potential fitness cost of insecticide resistance might also contribute to the maintenance of susceptible alleles into the population.

Additionally, our results revealed no known or putative novel mutations in any of the gene targets of pyrethroids and oxadiazines (VGSC), spinosad (nAChR_α6), diamides (RyR),

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Table 2. Log dose probit mortality data for *L. botrana* populations from Turkey

Strain	*N*	LC_{50}	CL 95%	RR		LC90	CL 95%	Slope	s.e.	H
Indoxacarb										
S	120	0.35	0.15–0.82		*A*	21.73	5.35–531	0.71	0.15	0.57
R	120	10.3	7.41–16.2	30	*C*	42.79	24.4–127	2.08	0.38	0.45
MA	120	1.37	0.90–1.94	4	*B*	6.82	4.38–14.6	1.84	0.31	0.24
Mah	120	8.73	6.36–11.8	25	*C*	23.7	16.2–54.1	2.95	0.65	0.34
MS	120	9.58	6.38–17.3	28	*C*	63.3	29.9–284	1.56	0.29	0.41
D	120	5.34	2.99–11.9	15	*C*	116	34.9–2641	0.96	0.23	0.01
Spinosad										
S	120	0.27	0.15–0.47		*A*	3.97	1.81–15.4	1.09	0.18	0.42
R	120	15.64	9.25–41.7	59	*D*	143	50.2–1692	1.33	0.29	0.29
MA	120	1.44	0.89–2.09	5	*B*	8.87	5.39–21.4	1.62	0.28	0.42
Mah	120	7.29	5.05–10.8	27	*cd*	29.4	17.7–83.8	2.12	0.43	0.30
MS	120	4.96	3.54–7.19	19	*C*	24.0	14.5–56.6	1.87	0.29	0.24
D	120	3.16	1.45–6.88	12	*bc*	120.1	30.6–7825	0.81	0.22	0.01
Deltamethrin										
S	120	1.22	0.66–2.82		*a*	24	7.84–218	0.99	0.18	0.12
R	120	36.57	19.8–72.5	30	*b*	216	98.8–1602	1.66	0.27	1.21
MA	120	26.28	18.1–37.9	22	*b*	145	87.9–342	1.73	0.27	0.64
Mah	120	21.66	14.8–30.2	18	*b*	87.6	57.6–177	2.11	0.35	0.21
MS	120	23.39	16.2–33.0	20	*b*	117	73.7–256	1.83	0.29	0.41
D	120	31.05	16.9–57.7	26	*b*	203	94.6–1275	1.57	0.26	1.04

S, Kahramanmaraş strain (susceptible reference strain); R, Manisa-Merkez strain; MA, Manisa-Alaşehir strain; MAh, Manisa-Ahmetli strain; MS, Manisa-Saruhanlı strain; D, Denizli strain; *N*, number of larvae; LC50, lethal concentration, expressed in ppm (95% confidence intervals); H, heterogeneity; RR, resistance ratio.
*Different letters indicate significant differences in the responses ($P < 0.05$).

avermectins (GluCl), and organophosphates (ace), in all populations tested. These indicated a low risk for the future selection of target site resistance in this region.

Despite the absence of resistance mutations, the bioassays indicated resistance against indoxacarb and deltamethrin for some populations from Turkey, possibly indicating alternative resistance mechanisms. This is in contrast to other lepidopteran species, such as *Tuta absoluta*, *Plutella xylostella*, and *H. armigera*, where target site mutations constitute the major resistance mechanism responsible for high resistance levels (reviewed by Guedes *et al.*, 2019; Banazeer *et al.*, 2022).

P450s have been found to play major roles in the insecticide resistance of lepidopteran species (Katsavou *et al.*, 2022), more specifically, 56 P450s have been validated for their contribution to resistance in ten economically important lepidopteran pest species. Here, through transcriptome assembly we identified 161 *L. botrana* CYPs, enzymes that are involved in key physiological processes. It is known that CYPs that are involved in ecdysteroid metabolism, a key physiological process in the moulting insects (Feyereisen, 2012), belong to Clan 2 and M and include the CYP302A, CYP306A, CYP307A, CYP314A, CYP315A, and CYP18A/B subfamilies. It is worth noting that we could detect orthologs of all these CYPs (fig. 3). Importantly, the lepidoptera-specific duplication of CYP18 is present as full-length transcripts. Also, CYP306A1 and CYP307A2 were both detected. In Clan M, there are two CYP302A1 fragments that most probably are different parts of the same transcript. In the same clan, there are also two CYP314A1-like fragments, whereas no CYP315A1 homologue was found. However, given the critical role of CYP315A1 it is possible that the reason we could not detect it is due to a low level of transcription, rather than it being indeed absent from *L. botrana*.

Another key physiological process is the biosynthesis of cuticular hydrocarbons. CYPs from the CYP4G subfamily (Clan 4) play an important role in this process. *H. armigera* contains five CYP4G genes and *L. botrana* contains four (fig. 3), only one of which, however, appears to be full-length. It is not easy to determine whether any of the three remaining CYP fragments originate from the same transcript. As a result, it is not easy to estimate the total number of CYP4G genes in the grapevine moth.

Our phylogenetic analysis showed that there are some notable expansions (blooms) in certain CYP subfamilies in *L. botrana*, such as in CYP333B, CYP341B, CYP6B, CYP6AB, and CYP338A (fig. 3 – annotated with a '+' inside a blue circle). More specifically, CYP333B3 (Clan M) is the only bloom in Clan M and it appears that the grapevine moth has at least three copies, compared to only one in *H. armigera*. This gene has also been extensively duplicated in another lepidopteran species, the tobacco hornworm *Manduca sexta* (Dermauw *et al.*, 2020). However, the function of the extra CYP333B copies has not been elucidated yet. CYP341B genes in Clan 4 are involved in the biosynthesis of sex pheromones and are frequently

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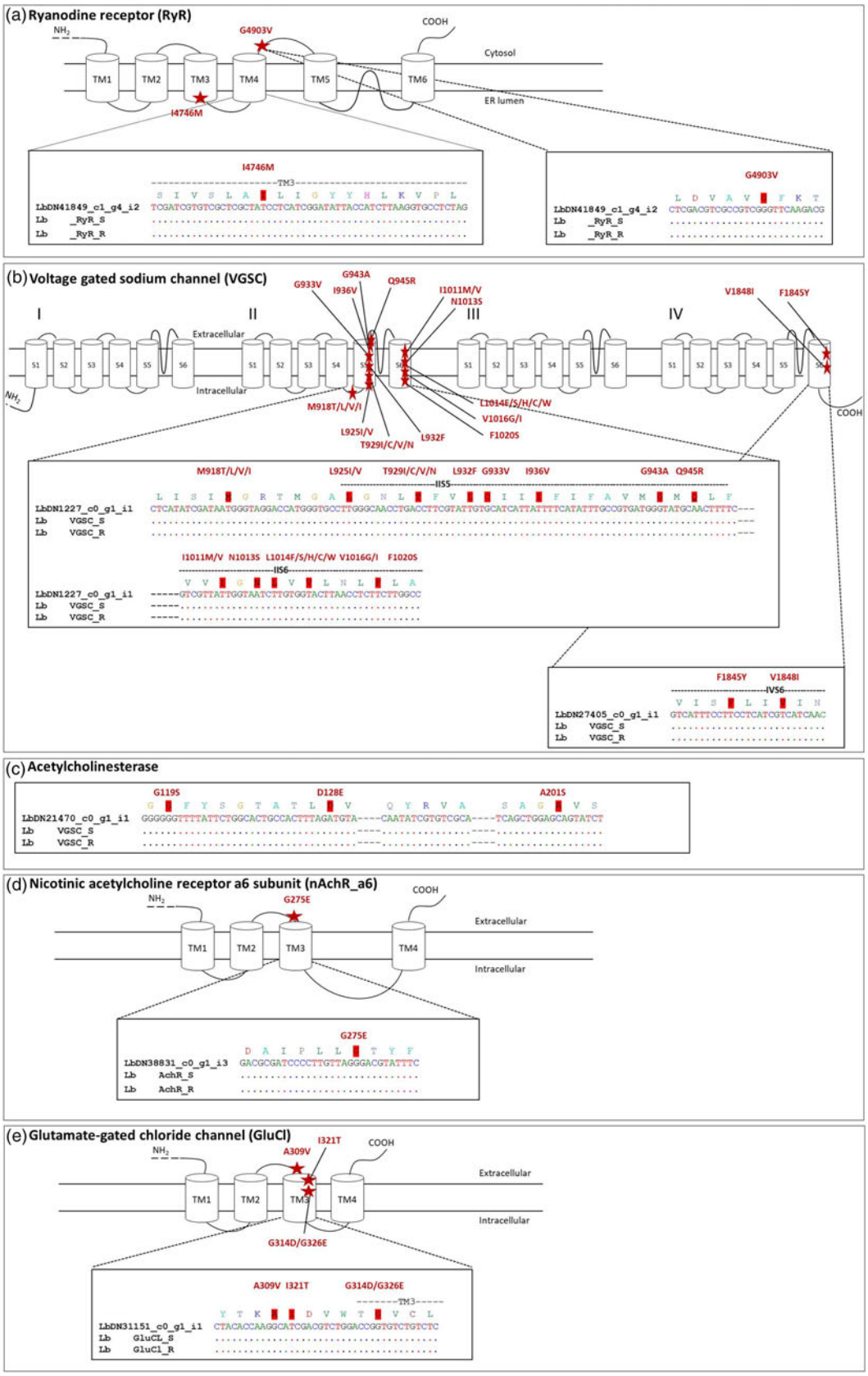

Figure 2. Target site mutations linked to insecticide resistance in arthropod species, with the corresponding alignment in *L. botrana* populations. Five different target genes were checked for mutations implicated in resistance against six insecticides. No mutations were detected in any of the 23 positions (amino acids highlighted in red). The structure of the transmembrane domains (except for acetylcholinesterase) is shown for each gene and red stars mark the position in which each mutation occurs. Finally, the actual alignment of the relevant gene area is shown beneath each gene. The alignment contains three sequences; the consensus *L. botrana* sequence (i.e. the Trinity transcript), followed by the sequence of the susceptible (S) and resistant (R) strain, which were collected from different locations in Turkey.

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Figure 3. Cytochrome P450 phylogeny. Maximum likelihood phylogeny of the grapevine moth CYPs (names shown in red) compared to those of the cotton bollworm, *H. armigera* (names shown in blue). All four insect CYP clans are well-supported with bootstrap values >65%. Branches from each of the four clans are coloured differently; Clan M – gold, Clan 2 – turquoise, Clan 3 – green, Clan 4 – orange. Full-length genes (>450 amino acids) are marked with a star, whereas those with a length between 250 and 450 amino acids are marked with a triangle. CYP clades that have bloomed in *L. botrana*, compared to *H. armigera* are annotated with a '+' inside a light blue circle, whereas contractions are annotated with a '–' inside a light red circle.

duplicated in Lepidoptera (Dermauw *et al.*, 2020). In the grapevine moth, there are as many as 23 *L. botrana* CYP341B genes that cluster with the five CYP341B genes of *H. armigera*. Two of these sequences are full-length genes and an additional eight are of intermediate length (between 250 and 450 amino acids, marked with a triangle in fig. 3), indicating that most probably there are more CYP341B copies in *L. botrana* than in *H. armigera*. Lepidopteran CYP341B genes are involved in the biosynthesis of sex pheromones (Dermauw *et al.*, 2020). As a result, an expansion CYP341B in *L. botrana* could possibly underlie behavioural differences between *L. botrana* and *H. armigera*.

The remaining three blooms are in Clan 3 subfamilies. The CYP6B clade appears to be greatly expanded in *L. botrana* with at least seven full-length genes and another 12 fragments, compared to only five genes in *H. armigera*. Additionally, the grapevine moth appears to have a great number of genes in the CYP6AB/6AN clade. More specifically, while there are only four 6AB and one 6AN genes in *H. armigera*, there are 27 CYP6AB (nine of which are full-length) and four CYP6AN genes in *L. botrana*. The *L. botrana* CYP6AB genes are grouped into three well-supported clades. All four *L. botrana* CYP6AN genes are sister to the CYP6AN1 of *H. armigera*, thus suggesting a possible duplication in the *L. botrana* lineage. Quite a number of CYP6B and CYP6AB genes have been associated with insecticide resistance (Katsavou *et al.*, 2022), and as a result the observed expansions could be responsible for insecticide resistance in *L. botrana*.

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Finally, there is a striking contraction in the CYP340 family (fig. 3 – noted with a '−' inside a red circle), whereby *L. botrana* appears to have only three genes, only one of which is nearly full-length. In sharp contrast, *H. armigera* has as many as 25 CYP340 genes grouped in seven different subfamilies (340Q, K, H, G, AD, AG, and J). This finding is in agreement with the previously reported recent expansion of the CYP340 family in *H. armigera* and *H. zea* (Pearce *et al.*, 2017). However, since the *L. botrana* genes could not be classified into a specific CYP340 subfamily (fig. 3) their role is not clear.

The assembled transcriptome, the set of unigenes and the annotation of cytochrome P450s generated in this study are publicly available in the NCBI TSA database and the Supplement of this manuscript, and they will facilitate molecular studies on *L. botrana*.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485323000640.

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