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Rapid molecular DNA identification method for the European invasive grapevine moth *Lobesia botrana*

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Executive summary

The aim of this project was to develop a robust, rapidand easy-to-use molecular identification method for the invasive European Grapevine Moth (EGVM) *Lobesia botrana*, a species which is a high priority exotic pest threat for the Australian grape and wine industry, and is difficult to identify visually. Initial work involved characterisation of the complete mitochondrial DNA (mtDNA) genome of EGVM, from which Polymerase Chain Reaction (PCR) primers were designed to amplify regions of the genes cytochrome oxidase I (COI) and cytochrome b (Cytb).Using these primers together with DNA sequencing allowed a survey of the genetic diversity of this pest moth, testing over 400 individuals sourced from both its native range across the Mediterranean basin and Asia, and from New World countries representing its recent invasive range. Extensive phylogenetic analysis based on mtDNA haplotype signatures suggests that the presence of EGVM in the Americas resulted from multiple incursion events or a single event involving at least four different maternal lineages.

Use of the COI PCR primers in combination with restriction fragment length polymorphism (RFLP) analysis was able to readily distinguish individuals of *L. botrana* from those of other *Lobesia* species. However, it is recommended that DNA sequencing on the PCR amplicon be carried out to ensure accurate species identification. A DNA extraction method was refined, which can be used to obtain DNA from EGVM individuals at any stage of the life cycle (egg, larva, pupa or adult), and a simple preservation method for field-collected samples was also developed. These procedures were combined to form a National Diagnostic Protocol for molecular identification of the European Grapevine Moth *Lobesia botrana*, which currently awaits review. Once nationally endorsed, this protocol will allow identification of EGVM in the event of an incursion and facilitate a rapid response by the grape and wine industry.

Part I Introduction

1 Introduction

Australia's wine industry is estimatedto be worth AUD$4.3 billion and to provide AUD$43 billion economic value to Australia (Wine Australia Annual Operation Plan 2013-2014). As Australia's fourth biggest agricultural exporting sector and with a world reputation to protect, understanding biosecurity threats to the industry and the ability to provide early detection is imperative. Of the invasive insect pests identified as posing a serious threat to Australia's table grape and wine grape industries, the European grape vine moth (EGVM) *Lobesia botrana*([Denis &Shiffermüller]) (Lepidoptera: Tortricidae: Olethreutinae), is regarded as one of the most significant viticulture pests. Globally, at least 104 named *Lobesia* species are known, however members within this genus are not well-studied. Native to the southern European/Asia/Asia Minor regions, this species has invaded various countries in Africa (e.g., Kenya, Ethiopia; CABI 2016) the US, Argentina and Chile (Gilligan et al. 2011; CABI 2016), and has the potential to establish in Australia due to the similar Mediterranean climatic conditions as in southern Europe, and the widespread availability of host cropsincluding grapes.The extent of population spread upon successful incursion has not been modelled specifically on Australia's unique and changing climatic patterns. A National Diagnostic Protocol (NDP) to identify *L. botrana* at all life stages will increase Australia's biosecurity preparedness that underpins plant health in vineyards, and can be readily developed via molecular DNA identification methods. Currently, no such methodology is available and this represents a significant weakness in Australia's biosecurity tool-boxto safeguard a valuable industry, thereby limiting the industry's ability to respond to incursions and to monitor spread in a timely manner.

The use of mitochondrial DNA (mtDNA) as a species identification tool has been well-documented in a wide range of organisms including the Lepidoptera (e.g., Behere et al. 2008). Development of a species identification method using PCR and restriction fragment length polymorphism (RFLP) analysis, or direct Sanger sequencing of PCR amplicons, will require detailed understanding of population-wide genetic diversity.Such a study will enable development of robust DNA markers and selection of the most appropriate restriction enzyme(s). Currently, there is no reliable public mtDNA database for *Lobesia botrana*. Until recently (21-Sept-2015), four partial *L. botrana* mtDNA cytochrome oxidase I (COI) sequences from four individuals representing the native European region (e.g., Netherlands, Germany, Greece) were reported in the International Barcode of Life (iBoL) DNA database. However, these have since been removed (last access date: 16-Jan-2016) due to concerns regarding the accuracy of the species nomenclature (P Hebert,pers. comm.). Partial mtDNA COI gene sequences from a total of 18 *Lobesia* species were also available in the public DNA databases. These included nine *Lobesia* species sampled from Australia, and nine from various world regions.

By widely sampling *L. botrana* populations from both its known native range and range representing recent invasive population expansion, this project will survey regions of *L. botrana* mtDNA COI and cytochrome b (cytb) gene diversity to generate a mtDNA haplotype database that

will enable effective differentiation of the invasive EGVM from various exotic and endemic *Lobesia* species. The EGVM genetic database will also provide fine-scale population information to assist with tracing possible population origins should there be incursions of EGVM in Australia. The project will adopt next generation sequencing (NGS) and standard Sanger sequencing technologies to both rapidly generate selected full mtDNA genomes of *L. botrana*, and conduct population-wide survey of mtDNA diversity that will be crucial to developing an accurate species identification method.

Results from this research will contribute to the AGWA Strategic RD&E Plan 2012-17 objectives of Plant Biosecurity and Education, and aligns with the 'Safeguarding Australia' national research priority. The project aims to develop a National Diagnostic Protocol (NDP) for *L. botrana* based on molecular DNA characters to rapidly confirm detection of the target pest species at all life stages (eggs, larvae, pupae and adults), thereby: (i) by-passing the need to rear eggs to larval stages and pupae to adulthood for positive identification; (ii) reducing reliance on taxonomists to confirm target pest species via morphological characters; (iii) providing a molecular signature to infer possible country of origin(s) and where the breach to biosecurity procedures may have occurred, and (iv) contributing to the training of researchers in diagnostic identification procedures for exotic pests.

2 Material and Methods

2.1 *Lobesia botrana* sampling sites and sample details

Adult and larval *Lobesia botrana* samples were collected from various vineyard locations in France (15 locations), Switzerland (3 locations), Portugal (4 locations), and Italy (1 location). Samples representing *L. botrana'*sinvasive range were also obtained from Chile (5 locations, all as pupae), and from USA (1 laboratory maintained population originally established as a mixed field-collected populations of >1,000 individuals) (Table 1, Fig. 1). The laboratory *L. botrana* samples provided by USDA were as eggs, larvae or pupae. All field-collected individuals used a simple and effective ethanol preservation method developed specifically for this study (Appendix A).In addition to these samples, extracted genomic DNA (gDNA) from Germany (2 populations), Turkey (2 populations), Israel (1 population), Syria (1 population), Italy (2 populations), Spain (1 population) that were previously used in a population study based on microsatellite DNA markers (Reineke et al. 2015) were also obtained from the corresponding author. Sampling dates, site details and life stages of the samples used in this study are provided in Table 1. Once received at the CSIRO Black Mountain Laboratories, all samples were kept at -20˚C until needed in the gDNA extraction step and/or PCR and sequencing.

Table 1: Sample details of *Lobesia botrana* used in in this study. Chile and USA populations represent recent geographic range expansion by this invasive moth species. Sample life stages, where known, are provided. In addition to whole specimens, samples were also provided as pre-extracted genomic DNA (as detailed in Reineke et al. 2015) that originated either from adults or larvae and were indicated as such (Adult/Larva).

	Location	Population Code	Year	GPS	GPS	Life Stage	N
1	Portugal	PRT26	2014	41.1726722	-7.5269500	Adult	10
2		PRT27	2014	41.1540333	-7.6292778	Adult	13
3		PRT28	2014	41.2471806	-7.4819806	Adult	13
4		PRT29	2014	41.2057583	-7.4298333	Adult	1
5	Spain	EYEL[3]	2011	38.616983	-1.123653	Adult/Larva	15
6	France	FRA04	2014	43.675941	-3.504803	Adult	12
7		FRA05	2014	43.631766	-3.504803	Adult	11
8		FRA06	2014	44.7308611	0.5407222	Adult	10
9		FRA07	2014	44.7308611	0.5407222	Larvae	2
10		FRA08	2014	44.7564722	0.5504444	Adult	6
11		FRA11	2014	44.262858	0.287565	Larvae	11
12		FRA12	2014	44.224298	0.287565	Larvae	12

13		FRA13	2014	42.738567	3.00435	Larvae	12
14		FRA14	2014	42.768717	2.836783	Larvae	11
15		FRA15	2014	43.0786	2.222917	Larvae	12
16		FRA16	2014	43.074362	2.175668	Larvae	12
17		FRA21	2014	47.2156750	0.0559333	Adult	11
18		FRA22	2014	47.2125889	0.0436333	Adult	13
19		FRA23	2014	47.2264028	0.0786417	Adult	12
20		FRA31	2015	43.6541667	3.4515278	Pupa	4
21	Italy	ITA18	2014	43.2019139	10.5871833	Larva	11
22		IMEO	2010	45.6183270	12.4542600	Adult/Larva	16
23		IVAL	?	45.901453	11.995509	Adult/Larva	15
24	Switzerland	CHE19	2014	46.42269	6.275918	Adult	12
25		CHE20	2014	46.382598	6.1807	Adult	11
26		CHE30	2014	46.1761111	8.9336111	Adult	13
27	Germany	DLOR	2012	48.798704	9.703811	Adult/Larva	15
28		DKAI	2007	48.109993	7.664092	Adult/Larva	15
29	Israel	ILMG	2013	33.1164450	35.7695940	Adult/Larva	16
30	Syria	SYKA	2010	35.613413	36.560907	Adult/Larva	15
31	Turkey	TMMA	2012	36.969894	35.356379	Adult/Larva	13
32		TMCA	2012	38.630556	27.422222	Adult/Larva	16
33	Chile	#CHL38, CHL39, CHL40	2015	-33.8361111	-70.6416667	Pupa	3
34		‡CHL41, CHL43, CHL44	2015	-33.2844444	-70.8291667	Pupa	6
35		§CHL42, CHL45	2015	-33.0819444	-070.7469444	Pupa	5
36		†CHL46	2015	-33.0150000	-70.6675000	Pupa	3
37	USA	††Lab	2014	N/A	N/A	Pupa/larva/egg	18

Note: EYEL (Yelca, Murcia); IMEO (Meolo, Italy); IVAL (Valdobbiadene, Italy); DLOR (Lorch, Germany); DKAI (Kaiserstuhl, Germany); ILMG (Merom Golan, Israel); SYKA (Kafr, Syria); TMMA (Merkez, Turkey); TMCA (Cakalazmak, Manisa, Turkey). # Paine, RegiónMetropolitana, Chile; ‡ Lampa, RegiónMetropolitana, Chile; § Colina, RegiónMetropolitana, Chile; † Mostazal - Provincia de Cachapoal - Región de O'Higgins. †† *Lobesia botrana* samples from USA were from a laboratory colony initially established from field-collected pupae of approximately 1,000 individuals from various Californian vineyards in February 2010 (Nadel Hannah, APHIS, pers. comm. 15-Oct-2014). Details not available are indicated by 'N/A'.

2.2 Published and publicly available mtDNA COI partial gene sequences of Lobesia species

GenBank and the iBoL nucleotide databases were searched for *Lobesia* species partial mtDNA sequences, using the published *L. botrana* complete mtDNA COI gene from the full mitochondrial DNA genome (KP677508; Piper et al. 2015) as input query sequence. All available *Lobesia* species partial mtDNA COI gene sequences obtained (Table 2) were aligned using the CLC Sequence Viewer Version 7.6 sequence alignment option and selecting default settings. Aligned sequences were trimmed at the N- and C-termini to generate a *L. botrana* intra- specific anda *Lobesia* inter-specific sequence data set for phylogenetic analyses and for estimating nucleotide divergence distances. For both intra- and inter-species, unique mtDNA COI haplotypes were identified based on the assumption that sequences must differed from each other by at least one nucleotide polymorphism.

Table 2: Mitochondrial DNA COI partial gene sequences of *Lobesia* species obtained from the GenBank and iBoL DNA databases (accessed 21-Sept-2015), for the purpose of estimating intra- and inter-specific nucleotide divergence in the *Lobesia* genus, and within *L. botrana* species.

Lobesia sp.	Country	N	iBoL process ID/ GenBank Accession number
L. botrana	Netherlands	1	[1]NLLEA1226-13
	Germany	1	[1]GWORB3724-14
	Greece	2	[2]ITLP154-13, [2]ITLP153-13
L. vanilla	Kenya	9	[1]KJ592177, [1]KJ592383, [1]KJ592392, [1]KJ592150, [1]KJ592097, [1]KJ592200, [2]KJ592298, [2]KJ592310, [†]KJ592341
L. xylistis	Australia	6	[1]HM372972, [2]KF404901, [3]KF398348, [3]KF396328, [†]KF395501, [†]KF401541
L. physophora	Australia	9	[1†]KF403641, [2]GU688797, [3]HM372970, [4]GU688768, [5]KF402127, [6]KF397870, [7]KF399033, [†]KF402163, [†]KF397973
L. reliquana	Austria	1	[1]KM573424
	Finland	8	[1]HM874541, [1]HM872159, [1]HM875119, [1]HM875854, [1]HM876494, [2]HM873071, [3]HM876495, [3]HM876496
L. transtrifera /nr. *transtrifera*	Australia	7	[1]GU688763, [2]KF404301, [2]KF406197, [2]KF395986, [†]KF402901, [†]KF396367, [†]KF401583
L arescophanes	Australia	5	[1]GU688767, [2]GU688794, [†]KF400317, [†]KF400743, [†]KF401892
L. peltophora	Australia	3	[1]GU688769, [2]KF395899,[†]KF397931
L. semosa	Kenya	3	[1]KJ592416, [1]KJ592407, [2]KJ592086
L. abscisana	Netherlands	1	[1]NLLEA1356-14
L. sp. A	Australia	4	[1]ANICB714-07, [2]KF399903 [†]KF402998, [†]KF405456

L. sp. D	Australia	1	[1]HM372971
L. sp. ANIC5	Australia	1	[†]KF397269
L. sp. ANIC6	Australia	1	[1]KF401250
'*Lobesia*'	Canada	7	[†1]KT128088, [†1]KT137943, [†1]SSGBC9460-14, [†1]KT128180, [†1]SSGBB4879-14, [†1]KT142605, [†1]KT148455
'*Lobesia*'	Papua New Guinea	4	[1]KP849919, [2]KP849984, [2]KP850656, [2]KP850076
L. virulenta	Austria	1	[1]KP150241
L. aeolopa	South Korea	2	[1]KF523801, [2†]KF523800

NOTE: Within each *Lobesia* species, identical super-script numbers indicates mtDNA COI iBoL/NCBI sequences that are identical. All sequences are aligned and trimmed at 5' and 3' end to 655bp that corresponded to nucleotide positions (nt) 40 to 694 of the *L. botrana* reference mitogenome KP677508. '†' indicates mtDNA COI sequences that have been excluded due to insufficient sequence length (i.e., <655bp).

2.3 Genomic DNA extraction

Genomic DNA (gDNA) from individual *L. botrana* larvae, pupae and adult moths were extracted following the method as described in the National Diagnostic Protocol (NPD) for *L. botrana*developed (Appendix B). Briefly, each individual was placed in a sterile 1.5mL Eppendorf tube and digested for 2-16 hours using the QiagenDNeasy Blood and Tissue DNA extraction kit (cat. No. 69506) with subsequent washes in a spin column followed instructions as provided by the manufacturer. Extracted gDNAwas eluted in 50µL of EB (Qiagen, Cat. No. 19086) and kept frozen at -20˚C until needed.

2.4 Complete Mitochondrial DNA genome Sequencing

A random individual of *L. botrana* (FRA_15-1) was selected and its extracted gDNA was prepared into a Nextera genomic DNA library for Illumina MiSeq sequencing (Piper et al. 2015). Construction of the gDNA library followed the protocol as provided by Illumina. The gDNA library was subsequently sequenced to obtain the full mitochondrial DNA genome (mitogenome) ofthis individual for the purpose of developing effective PCR and Sanger sequencing primers. Additional nine random *L. botrana* individuals were also sequenced using the Illumina MiSeq NGS platform to generate their mtDNA genomes (Table 3) following the Piper et al. (2015) method described. An individual from France (sample code FRA_23-10) was found to have significantly different mtDNA COI partial gene sequence from the all other *L. botrana* samples, and the mitogenome of this unique individual was therefore also sequenced.

2.5 Mitogenome assembly and annotation

Assembly of the mitogenome of FRA_15-1 individual has been reported in Piper et al. (2015). Assembly of mitogenomes from nine additional EGVM individuals from MiSeq sequence reads included individuals from France(FRA_16-01, FRA_04-02, FRA_06-01, FRA_12-01, FRA_14-01), Portugal (PRT_26-01), Germany (DLOR_05), and Israel (ILGM_09, ILGM_15) (Table 2). Annotation of mitogenomes for these nine individuals was based on the annotated mitogenome of the *L. botrana* individual (KP677508) published by Piper et al. (2015). The single individual of *Lobesia* species from France (FRA_23-10) that showed substantial sequence variation was also sequenced using the Illumina MiSeq platform and assembled using Geneious® 8.0.5 (Biomatters Ltd., Auckland, N.Z.), and annotated using MITOS<http://mitos.bioinf.uni-leipzig.de/index.py>(Bernt et al. 2013)as described in Piper et al. (2015). Fine scale manual adjustments for annotation of mitogenomes post MITOS analysis were performed within Geneious® 8.0.5, and included manually annotate and checking that all protein coding genes (PCGs)included putative start codon ('M', methionine) and stop codons. Identification of the start codon was achieved by translating all PCGs to amino acids based on the invertebrate mitochondrial genetic code (code 5) Geneious® 8.0.5. All anticodons for transfer RNAs (trn) were as identified by MITOS based on the 'invertebrate mitochondrial DNA genetic code 5' mitogenome analysis option.

Table 3: Sample details of *Lobesia botrana* individuals sequenced for full mitogenomes, and included one potential 'cryptic' *L. botrana* individual (sample code FRA_23-10; GenBankKX621053). Note that the complete mitogenome for the *L. botrana* individual (sample code: FRA_15-01) has been published (Piper et al. 2015).

SPECIES	SAMPLE CODE	COUNTRY	GPS	GPS	NOTES
Lobesia botrana	FRA_15-01	France	43.0786	2.222917	Piper et al. (2015)
Lobesia botrana	FRA_16-01	France	43.074362	2.175668	Unpublished
Lobesia botrana	FRA_04-02	France	43.675941	3.504803	Unpublished
Lobesia botrana	FRA_06-01	France	44.7308611	000.5407222	Unpublished
Lobesia botrana	FRA_12-01	France	44.2242980	000.2875650	Unpublished
Lobesia botrana	FRA_14-01	France	42.768717	2.836783	Unpublished
Lobesia botrana	PRT_26-01	Portugal	41.1726722	-007.5269500	Unpublished
Lobesia botrana	DLOR_05	Germany	48.798704	9.703811	Unpublished
Lobesia botrana	ILGM_09	Israel	33.1164450	035.7695940	Unpublished
Lobesia botrana	ILGM_15	Israel	33.1164450	035.7695940	Unpublished
Lobesia sp.	FRA_23-10	France	47.2264028	000.0786417	Unpublished

2.6 PCR/Sequencing primers design and optimisation

EGVM-specific PCR primers for partial mtDNA COI and Cytb gene regions were developed based on the complete *L. botrana*mitogenome as reported in Piper et al. (2015). PCR and Sanger sequencing primers were designed using the primer analysis software Oligo Version 7.60 (Moleuclar Biology Insights, Inc., Cascade, CO USA), and targeting the partial COI gene region that can enable inter-species comparison. To maximise the genetic resource for EGVM biosecurity preparedness, a second partial mtDNA gene Cytb region was also targeted with appropriate primers designed (Lb-Cytb-F01: GGATGATTAATTCGAACTTTACAC; Lb-Cytb-R01: GTTGAATATGTGGAGGAGTTAC). All PCR and sequencing primers were developed following criteria and methods as previously reported in Tay et al. (2008). Briefly primers were developed with minimal duplex formation, false priming sites, primer dimer and hairpin loop formation, and with a theoretical annealing temperature of [2˚C x (A+T) + 4˚C x (G+C)] ≥ 60˚C. Optimisation of PCR primers was carried out on a Bio-RAD thermal cycler Series 1000 with gradient temperature profile of 48˚C to 58˚C. PCR conditions for Cytb region were as the COI gene region PCR conditions. The primer pairs for the COI gene region are as detailed in the *L. botrana* NPD protocol (Appendix B).

2.7 Sanger sequencing, trace file analyses and contig assemblies

All methodologies for Sanger sequencing of PCR amplicon, and post sequencing reaction clean-up have been detailed in the NPD prepared for *L. botrana* (Appendix B).Sanger sequencing was carried out by the Australian National University Biological Resource Facility at the John Curtin School of Medical Research, Canberra, Australia. Sequence trace files were analysed for sequencing errors prior tocontig assembly using the StadenPregap and Gap4 programs (Staden et al. 2000). All contigs assembled were checked for possible presence of pseudogenesby translating into amino acid sequences to ensure no premature stop codons prior to being entered into the CLC Sequence Viewer Version 7.6 (Qiagen Aarhus A/S) stand-alone DNA database for downstream analyses.

2.8 DNA data analyses

2.8.1 *Phylogenetic analyses of Lobesia species based on mtDNA COI*

To better understand the phylogenetic position of EGVM with respect to related *Lobesia* species for which mtDNA COI sequences were available from iBoL and GenBank databases, a Maximum Likelihood phylogeny was constructed using the web-based phylogenetic analysis program PhyML<http://www.atgc-montpellier.fr/phyml/> (Guindon et al. 2010), and selecting the automatic estimate option for estimating the most appropriate evolutionary rate parameters and included 500 bootstrap replications to estimate node confidence.Similarly, intraspecific phylogenetic relationships across different *L. botrana* maternal lineages were also inferred from the mtDNA COI partial gene sequences using PhyML and selecting the same automatic options to

estimate most appropriate evolutionary model and rate parameters, as well as inferring node confidence by 500 bootstrap replications. Phylogramsobtained (showing ≥50% node confidence values) for both intra- and inter-specific analyses werevisualisedand manipulated using the software Dendroscope (Huson et al. 2007).

2.8.2 AMOVA, and Inter- and Intra-specific sequence divergence estimates

An Analysis of Molecular Variance (AMOVA) based on concatenated partial COI and Cytb genes at the population-wide level was carried out within the PopArt package <http://popart.otago.ac.nz> for all *L. botrana* individuals. The molecular analysis software package MEGA6 (Tamura et al. 2013) was also used to estimate inter- and intra-specific nucleotide divergence values by selecting the best nucleotide substitution model and gamma-shape parameters as obtained from the PhyML analyses described above.

2.8.3 Mitochondrial DNA haplotype networks and global haplotype patterns

All *L. botrana*individuals with unique mtDNA COI and Cytb haplotypes from across the sampling range were used to constructa mtDNA COI and Cytb concatenated gene-specific haplotype network. To assist with data visualisation, haplotype networks and haplotype proportions for individual countries sampled were prepared using the software PopArt<http://popart.otago.ac.nz>. Where necessary the generated haplotype networks were manually rearranged to aid with visualisation. A global *L. botrana* population-wide mtDNA haplotype pattern, based on concatenated partial mtDNA COI and Cytb gene sequences, was also prepared. The table lists all unique haplotypes detected for all *L. botrana* individuals according to geographic locations, and will enable matching of haplotypes detected in new expansion ranges to historical locations.

2.8.4 PCR-RFLP development and gel electrophoresis

All partial mtDNA COI unique sequences generated by this study were aligned with all available *Lobesia* species from public DNA databases and subject to *in silico* RFLP analyses involving all commercially available restriction enzymes. *In silico* RFLP analyses were carried out using two analysis packages: CLC Sequence Viewer Version 7.6 RFLP analysis, followed by reconfirmation of selected restriction enzymes and estimation of fragment sizes using the program Enzyme X version 3.3.3.<http://nucleobytes.com/enzymex/>. Restriction enzymes ascertained as being most promising were purchased from commercial suppliers and used to test predicted digestion patterns on PCR amplicons using specifically re-designed PCR-RFLP primers, and following the reaction protocols and incubation conditions as instructed by the suppliers (see Appendix B). PCR-RFLP digested amplicons were run on a 1.25% 1xTAE agarose gel against a 100bp DNA size marker ladder (NEB, Cat. # N3231S) at 90V for 90 minutes. Visualisation of the

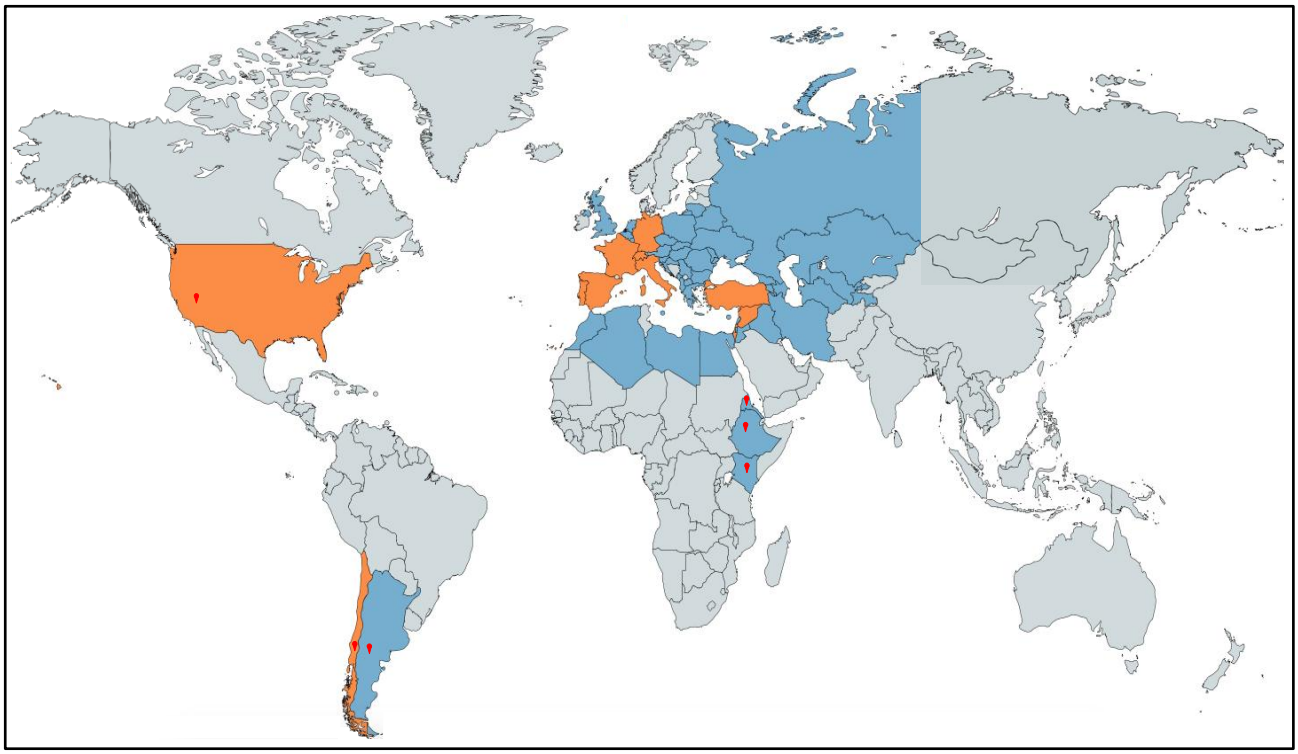
RFLP patterns was carried out using a UV-transilluminator fitted with a gel documentation system. The exact protocol for the PCR-RFLP procedures has been detailed in the NDP (see Appendix B).

3 Results

3.1 *Lobesia botrana*sampling details

A total of 406 moths collected from 37 populations from across 11 countries were sampled (Table 1, Fig 1). These included two countries (USA, Chile) that represented *L. botrana*'s recent range expansion into the New World (Fig. 1). This data set also included material that represented all life stages of *L. botrana*, from eggs to larvae, pupae and adult moths. As the project included the aims of developing suitable protocols for handling of biological specimens representing specific life stages of the EGVM, gDNA extraction protocols were optimised to ensure successful extraction of high-quality gDNA. A complete step-by-step protocol for eggs and for larger insect material (i.e., larvae, pupae, adults) are provided in the *L. botrana*NPD (see Appendix B).

Figure 1: *Lobesia botrana* distribution range and sampling sites. Regions in blue represent current known distribution range of *L. botrana* that were not sampled, while regions in orange represented countries that were sampled for this study. Red markers in east Africa (Eritrea, Ethiopia, Kenya), North America (USA) and South America (Chile, Argentina) represent invasive range of the EGVM in recent times.



Note: Distribution of *L. botrana* beyond central Russia is unknown and is therefore not highlighted.

3.2 Mitogenome annotation and diversity

The annotated mitogenomes of the 10 randomly selected *L. botrana* individuals all exhibited same numbers of protein coding gene (n=13), transfer RNAs (trn; n=22), ribosomal RNA (rrn; n=2), as well as gene orientations including locations of the putative replication origin. A diagrammatic representation of the EGVM mitogenome is provided (Fig. 2). The mitogenomes of individuals from France (n=6), Germany (n=1), Portugal (n=1), and Israel (n=2) were therefore similar in length, ranging from 15,196bp to 15,238bp, and exhibited similar GC content of between 19.9% and 23.3% (Table 4). Mitogenomes from French, Germany and Portugal individuals were overall the most similar to each other, while Israeli individuals were the most diverse (Appendix C). The suspected *L. botrana* cryptic species (Fra_23-10; GenBank accession number KX621053) had a mitogenome size of 15,224bp and is within the range detected for *L. botrana*, however it has a very different GC content of 29% (Table 4). All unpublished *L. botrana* and the *Lobesia sp*. (Fra_23-10) mitogenomes shared the same protein coding gene, rRNA and tRNA gene features, consisted of 13 protein coding genes, 2 ribosomal RNA genes (16S and 12S), and 22 transfer RNA genes, and a putative replication origin (also referred to as A+T rich region, or 'D-loop').

Table 4: Summary table of general mitogenome characteristics between the ten *L. botrana* individuals and also between the likely cryptic *L. botrana* individual (FRA_23-10;KX621053). The lengths of the mitogenomes (in base pairs, (bp)) and GC contents (in %), as well as specific country of origins and sample codes are provided.

LOBESIA SPECIES	SAMPLE CODE	COUNTRY	LENGTH (bp)†	GC (%)	NOTES
Lobesia botrana	FRA_15-01	France	15,229	19.90	Piper et al. (2015)
Lobesia botrana	FRA_16-01	France	15,231	20.00	Unpublished
Lobesia botrana	FRA_04-02	France	15,229	19.90	Unpublished
Lobesia botrana	FRA_06-01	France	15,229	19.90	Unpublished
Lobesia botrana	FRA_12-01	France	15,228	19.90	Unpublished
Lobesia botrana	FRA_14-01	France	15,196	20.00	Unpublished
Lobesia botrana	PRT_26-01	Portugal	15,238	20.30	Unpublished
Lobesia botrana	DLOR_05	Germany	15,225	20.05	Unpublished
Lobesia botrana	ILGM_09	Israel	15,229	23.30	Unpublished
Lobesia botrana	ILGM_15	Israel	15,226	20.70	Unpublished
Lobesia sp.	FRA_23-10	France	15,224	29.00	Unpublished

Note: (†) final mitogenome lengths should be regarded as an approximation, due to potential assembly errors associated with PCR amplification errors across low nucleotide complexity regions such as intergenicregions and the putative replication origin / A+T rich region.

The number of single nucleotide polymorphisms (SNPs) observed between the ten mitogenomes of *L. botrana* varied between the 13 protein coding genes, ranging from 0 in the ATP8 gene to 20 in NAD5, while the average frequency of SNPs observed per nucleotide protein coding gene varied between 0 (in ATP8) to 0.0269 (NAD4L) (Table 5). The SNP frequency of the

widely used mtDNA COI gene was amongst one of the lowest, being similar to NAD5, NAD1 and COII, while ATP6 (0.0162), NAD6 (0.0171) and NAD4L (0.0269) were the highest. Of the 22 tRNAs present, four (trnA, trnN, trnS2 and trnV) each had one SNP and the trnL1 gene had two INDELs. INDELs and SNPs were also identified in the two rRNA genes, as well as in the intergenic /A+T rich (i.e., putative origin of replication, or D-loop region). However, sequence variation identified in the A+T rich region should be interpreted with caution because of difficulties with the assembly of low complexity genomic regions, such as the A+T rich region in mitogenomes (Piper et al. 2015). Schematic representation of a complete *L. botrana* mitogenome is presented in Fig. 2, and comparative annotations of complete mitogenomes between a *L. botrana* and the potential cryptic species are tabulated in Table 6. The full mitogenome sequence of the cryptic/misidentified *L. botrana* individual (FRA_23-10) has been deposited in GenBank(KX621053).

Table 5: Summary of mitogenome polymorphism patterns observed between ten EGVM individuals. Single nucleotide polymorphisms (SNPs) and nucleotide insertion/deletion patterns (INDELs) were scored for individual protein coding genes (PCG), transfer RNAs (trn), ribosomal RNAs (rrn), and for intergenic and the A+T rich regions. Gene lengths are as number of nucleotides (nt). Average SNPsatindividualPCGs were calculated as per nucleotide (SNP/nt).

	Gene	SNPs	INDELs	nt	SNP/nt	Category
1	COI	17		1536	0.0111	PCG
2	COII	7		717	0.0098	PCG
3	ATP8	0		162	0	PCG
4	ATP6	11		678	0.0162	PCG
5	COIII	11		789	0.0139	PCG
6	NAD3	5		357	0.0140	PCG
7	trnA	1		65	0.0154	tRNA
8	trnN	1		68	0.0147	tRNA
9	NAD5	20		1794	0.0111	PCG
10	NAD4	17		1404	0.0121	PCG
11	NAD4L	8		297	0.0269	PCG
12	Intergenic region	1				
13	NAD6	9		528	0.0171	PCG
13	COB	15		1149	0.0131	PCG
14	trnS2	1		65	0.0154	tRNA
16	NAD1	10		933	0.0107	PCG
17	trnL1		2	67	0.0299	tRNA
18	16s rRNA	8		1373	0.0058	rRNA
19	trnV	1		63	0.0159	tRNA
20	12s rRNA	2	1	777	0.0026	rRNA
21	A+T rich/intergenic region	7	7			
22	NAD2	13		975	0.0133	PCG

Note: no SNPs/INDELs were detected in the remaining tRNA units of the ten mitogenomesand are therefore not shown in the summary table.

Figure 2: Schematic representation of an annotated *Lobesia botrana* (KP677508; Piper et al. 2015) complete mitogenome showing 13 protein coding genes (PCGs, in yellow) including the direction of translation, 22 transfer RNAs (in pink), 2 ribosomal RNA (16S large subunit ribosomal RNA, 12S small subunit ribosomal RNA, red arrows), and the putative origin of replication (also known as the A+T rich / D-loop region, in grey).

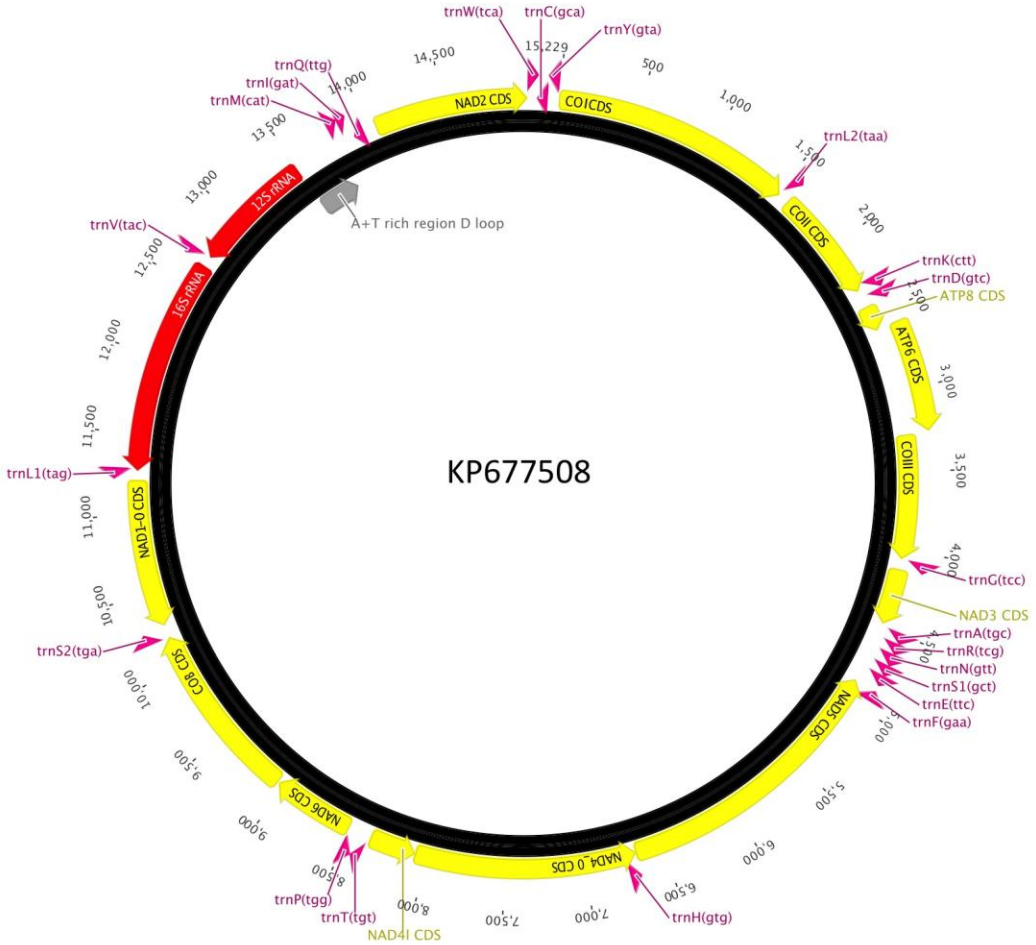


Table 6: Mitogenome annotations for *Lobesia botrana* (FRA_15-1, Piper et al. 2015) and a 'cryptic' *L. botrana* (unpublished, KX621053). Gene translation directions are indicated as 'forward' or 'reverse'. Anticodons for specific transfer RNAs are in parentheses, 'CDS' and 'D-loop' indicate protein coding genes and the A+T rich/putative replication origin regions.

		FRA_15-1 (KP677508)			FRA_23-10 (KX621053)			
Name	Type	Min	Max	Length	Min	Max	Length	Direction
COI	CDS	1	1,536	1,536	1	1,536	1,536	forward
trnL2(taa)	tRNA	1,532	1,598	67	1,532	1,598	67	forward
COII	CDS	1,600	2,316	717	1,600	2,316	717	forward
trnK(ctt)	tRNA	2,282	2,352	71	2,282	2,352	71	forward
trnD(gtc)	tRNA	2,365	2,430	66	2,359	2,424	66	forward
ATP8	CDS	2,431	2,592	162	2,425	2,586	162	forward
ATP6	CDS	2,586	3,263	678	2,580	3,257	678	forward
COIII	CDS	3,271	4,059	789	3,265	4,053	789	forward
trnG(tcc)	tRNA	4,062	4,126	65	4,056	4,122	67	forward
NAD3	CDS	4,124	4,480	357	4,120	4,476	357	forward
trnA(tgc)	tRNA	4,496	4,560	65	4,492	4,555	64	forward
trnR(tcg)	tRNA	4,561	4,626	66	4,556	4,622	67	forward
trnN(gtt)	tRNA	4,631	4,698	68	4,627	4,694	68	forward
trnS1(gct)	tRNA	4,702	4,767	66	4,695	4,760	66	forward
trnE(ttc)	tRNA	4,768	4,838	71	4,761	4,827	67	forward
trnF(gaa)	tRNA	4,872	4,935	64	4,862	4,926	65	reverse
NAD5	CDS	4,875	6,668	1,794	4,865	6,607	1,743	reverse
trnH(gtg)	tRNA	6,669	6,735	67	6,612	8,069	1,458	reverse
NAD4_0	CDS	6,672	8,075	1,404	6,665	6,730	66	reverse
NAD4l	CDS	8,077	8,373	297	8,069	8,362	294	reverse
trnT(tgt)	tRNA	8,388	8,451	64	8,383	8,446	64	forward
trnP(tgg)	tRNA	8,452	8,516	65	8,447	8,512	66	reverse
NAD6	CDS	8,520	9,047	528	8,515	9,042	528	forward
COB	CDS	9,057	10,205	1,149	9,052	10,200	1,149	forward
trnS2(tga)	tRNA	10,204	10,268	65	10,199	10,262	64	forward
NAD1-0	CDS	10,287	11,219	933	10,281	11,213	933	reverse
trnL1(tag)	tRNA	11,227	11,293	67	11,221	11,286	66	reverse
16S rRNA	rRNA	11,276	12,648	1,373	11,264	12,639	1,376	reverse
trnV(tac)	tRNA	12,652	12,714	63	12,642	12,705	64	reverse
12S rRNA	rRNA	12,715	13,490	776	12,705	13,468	764	reverse
D loop/A+T rich	misc	13,491	13,776	286	13,469	13,764	296	forward
trnM(cat)	tRNA	13,777	13,843	67	13,765	13,832	68	forward
trnI(gat)	tRNA	13,844	13,906	63	13,833	13,896	64	forward
trnQ(ttg)	tRNA	13,904	13,971	68	13,894	13,962	69	reverse
NAD2	CDS	14,056	15,030	975	14,046	15,020	975	forward
trnW(tca)	tRNA	15,033	15,099	67	15,022	15,090	69	forward
trnC(gca)	tRNA	15,092	15,153	62	15,083	15,146	64	reverse
trnY(gta)	tRNA	15,161	15,226	66	15,156	15,222	67	reverse

3.3 Interspecific mtDNA COI partial gene comparison

Partial mtDNA COI gene sequences for 18 *Lobesia* species were publicly available from the international Barcode of Life (iBoL) consortium. These sequences were generated from species that have been identified based on morphological characters and are often vouchered specimens deposited in various international insect collection facilities,including in the Australian National Insect Collection (ANIC) facility in Canberra, Australia.All known *Lobesia* partial mtDNA COI sequences (Table 2) were aligned to determine the degree of inter-specific variation. Sequences were trimmed at the 5' and 3' terminal regions to allow meaningful alignment and for phylogenetic inference.

3.4 Phylogenetic analysis of Lobesia species based on partial mtDNA COI gene

Based on partial (655bp) mtDNA COI gene of all *L. botrana* unique haplotypes and all publicly available *Lobesia* species, a Maximum Likelihood phylogenetic tree was constructed using *Endothenianubilana* (KM546492) and *E. nigricostana* (KM573203) (Lepidoptera: Tortricidae: Olethreutinae) as out-groups (Fig.3). The Phylogenetic analysis using PhyML 3.0 (Guindon et al. 2010) on all unique iBoL*Lobesia* species with sufficient (655bp) mtDNA sequence length (see Table2), and included all unique *L. botrana* sequences identified from population-wide survey (including also two iBoL sequences of ITLP154-13, ITLP153-13), and the potential *L. botrana* cryptic/misidentified species (FRA_23-10 (this study); NLLEA1226-13 (iBoL); GWORB3724-14 (iBoL)). The automatic evolutionary substitution model selection option available inPhyML 3.0 analysis identified the GTR+G6+I evolutionary model with gamma shape parameter =1.345, and proportion of invariable sites = 0.613 as being most suitable for the partial mtDNA COI sequence data set. All 20 *L. botrana* haplotypes were clustered together with a high bootstrap support value (96.6%) for the most recent common ancestor (red branch), while the potentially misidentified/cryptic*L. botrana* (FRA_23-10; including the other two individuals from iBoL (NLLEA1226-13 and GWORB3724-14); blue branch) shared a most recent common ancestor with *L virulenta* (KP150241) with 79.8% bootstrap support, and are both clustered as a sister-branch to *L. botrana*, adding support that FRA_23-10 and the iBoL sequences (NLLEA1226-13; GWORB3724-14) were likely to be a different species from*L. botrana*.

3.5 Intra-species phylogeny for Lobesia botrana

Within the*L. botrana* population surveyed, an un-rooted partial COI-Cytb concatenated gene Maximum Likelihood phylogeny (1,277bp) identified four major *L. botrana* clades (Fig. 4). PhyMLidentified the best nucleotide substitution model, substitution rate category, proportion of invariable sites and gamma shape parameter as TN93+G6+I, 0.488, 6, and 0.015, respectively. Bootstrap replications (500) were performed to ascertain node confidence, and bootstrap values of >50% are indicated for respective nodes. Overall, based on mtDNA partial COI and Cytb gene diversity, EGVM populations sampled could be broadly grouped into four clusters/clades (I, II, III,

IV; Fig. 4). Clade I contained predominantly the 'Israel/Syria' populations but also some Spanish haplotypes. Clade IV included mainly France populations but also haplotypes from neighbouring countries (i.e., Italy, Portugal, Switzerland). Clades II and III contained haplotypes that were predominantly from populations that originated from Portugal to Italy, Switzerland, Germany and Turkey. Clades II, III and IV also contained *L. botrana* haplotypes found in the New World invasive ranges of Chile and USA, while the two most common haplotypes ((02,11) and (18,11); Table 2) detected amongst surveyed populations were in clades III and IV. Three haplotypes ((18,01), (02,01), and (02,02)) were divergent from the haplotypes within the four clades and were from the France/Italy regions. The haplotype (20,20) appeared to be the most divergent, supportingthe hypothesis that it belongs to a different species of *Lobesia*.

Figure 3: Maximum Likelihood phylogeny (PhyML) of *Lobesia* species based on partial (655 bp) of mtDNA COI gene sequence. All *L. botrana* haplotypes identified in this study clustered within a single clade (shown in red), and included two iBoL sequences (ITLP153-13 and ITLP154-14) from Crete. The potential cryptic *L. botrana* individual from France (FRA_23-10; KX621031) shared the same haplotype as two other individuals reported in iBoL from the Netherlands (NLLEA1226-13) and Germany (GWORB3724-14) (blue branch), and are evolutionary more closely related to *L. virulenta*. Orange and blue arrows indicate other potential cryptic and poorly defined species within the *Lobesia* genus.

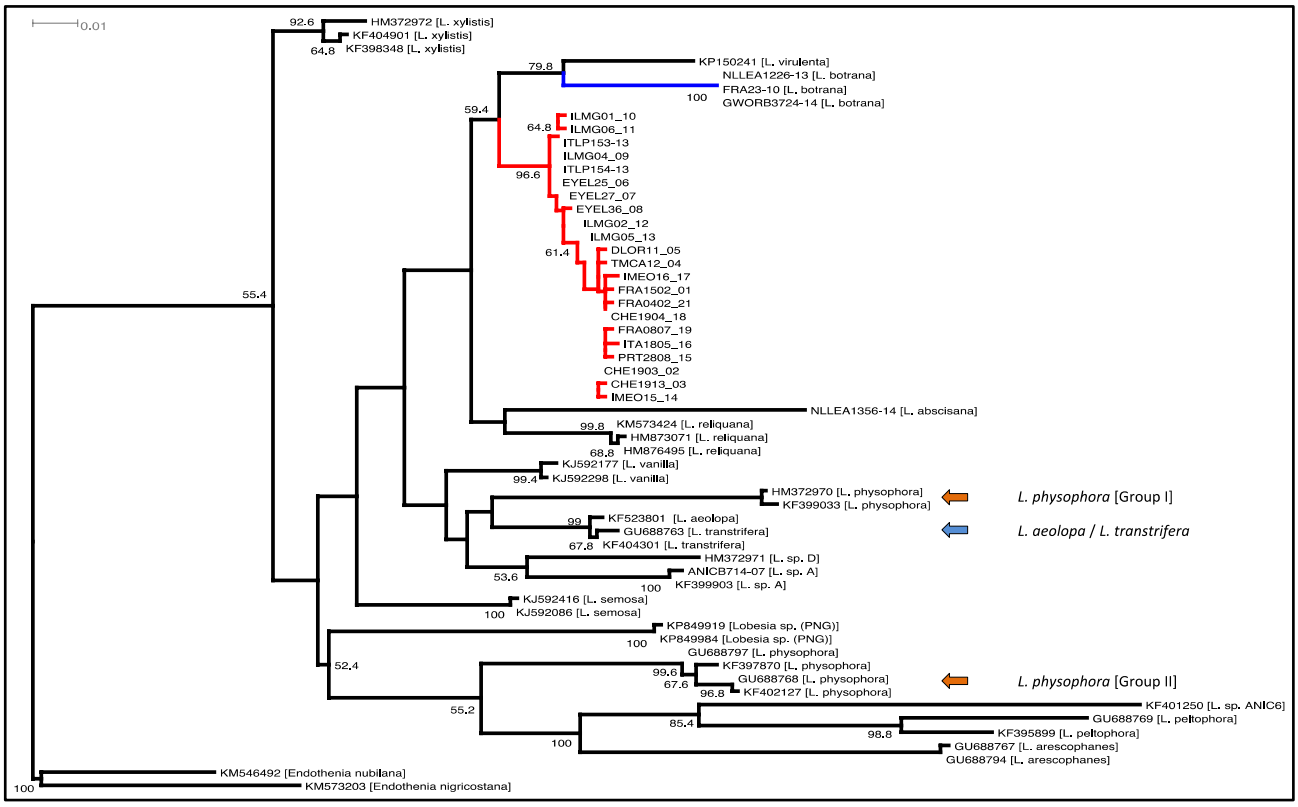
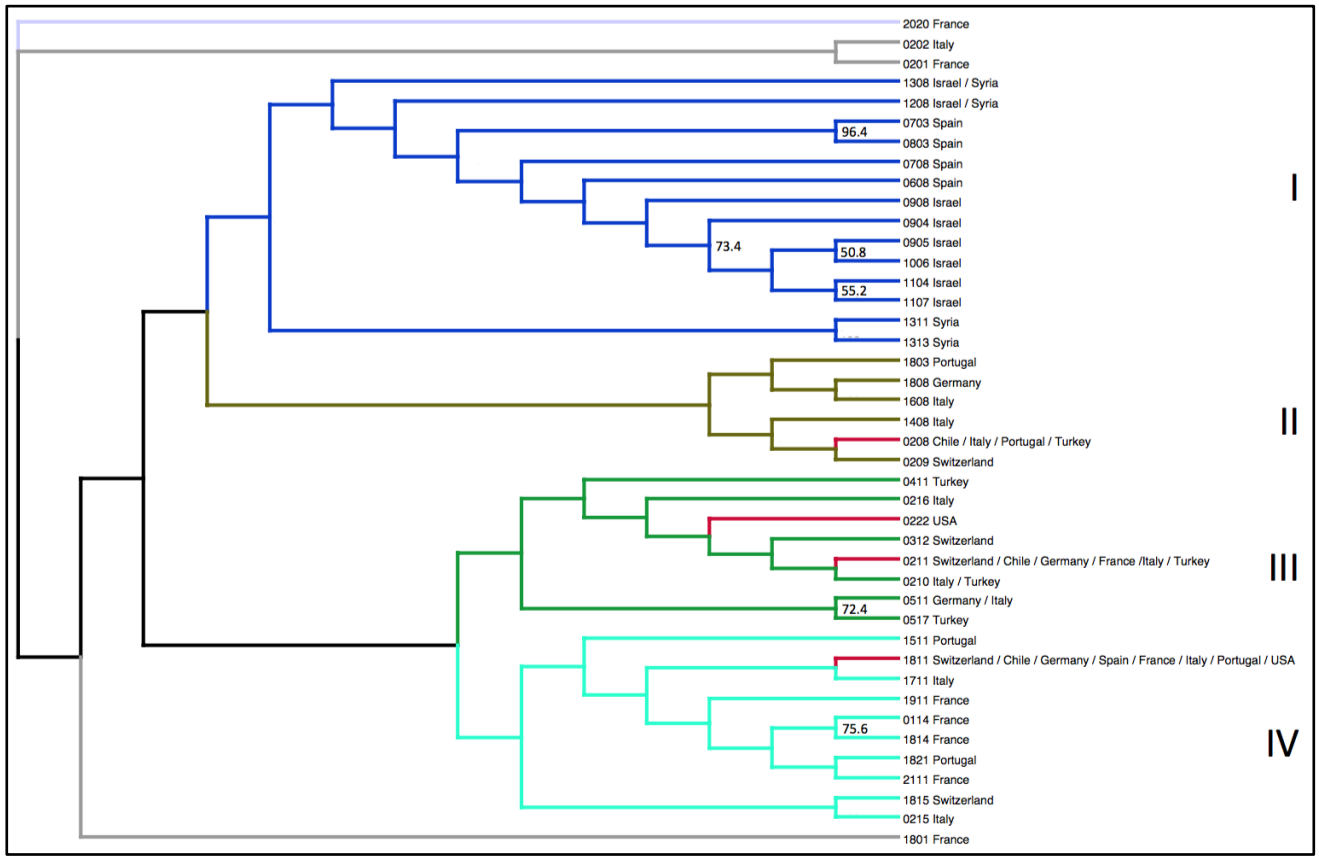


Figure 4: Maximum Likelihood phylogeny (PhyML) of *Lobesia botrana* unique mtDNA COI and Cytb concatenated haplotypes (1,277 bp). From a total of 42 haplotypes (see Table 5), four major sister clades (I, II, III, IV) were identified. Clade I contained predominantly 'Asian' haplotypes from Syria and Israel but also Spain. Clades II, III and IV contained the 'high frequency' haplotypes found in most sampling sites but also the haplotypes from New World populations (red branches). The French haplotypes were predominantly found in clades III and IV but also in minor (grey branches). The potential cryptic *L. botrana* individual with haplotype (20,20) was not clustered with any of the other *L. botrana* haplotypes. Bootstrap values >50% from 500 replications are indicated. Low bootstrap confidence values (<50%) at most branch nodes suggested presence of limited parsimony informative sites in the two-gene concatenated sequences.



3.6 AMOVA results

Analysis of Molecular Variance (AMOVA) is a statistical model for the molecular variation in a single biological species. AMOVAconsidering Europe, Asia and New World as hierarchical spatial groups produced a significant ϕ_{ST}value (0.108, P<0.001), indicating that significant variation exists between populations from different regions. Significant among-population variation (ϕ_{SC}; 0.079 P < 0.001) was also detected,suggesting that, on the basis of mtDNA variation, populations do not freely exchange individuals. At the within-population level, the amount of genetic variation observed was not significant (ϕ_{CT}; 0.031 P = 0.096) (Table 7).

Table 7: Analysis of Molecular Variance (AMOVA) of *Lobesia botrana* populations across different spatial structures from sampling regions.

Spatial Scale	Hierarchical levels	Variance	d.f.	% variation	Fixation indices	P values†
EU/Asia/New World	Among groups	0.708	2	3.099	0.108 ϕ_{ST}	< 0.001
	Among populations	1.755	14	7.678	0.079 ϕ_{SC}	< 0.001
	Within populations	20.392	389	89.223	0.031 ϕ_{CT}	0.096
	Total	22.855	405			

†Significance (1000 permutations) for random value > observed ϕ_{ST},ϕ_{SC}, and ϕ_{CT}, respectively.

3.7 Inter- and intra-specific nucleotide distances

Average interspecific nucleotide distances (P-dist with 500 bootstrap replications for standard error (s.e.) estimates) for 655bp COI partial gene region were estimated for all *Lobesia* species groups identified by the PhyML analysis (Table 8). Average mean distances ranged between 0.041 ± 0.007 s.e.(*L. vanillana* and *L. transtrifera*) and 0.125 ± 0.0013 s.e.(*L.* sp. ANIC 6 and *L. reliquana*). Between *L. botrana* and the possible cryptic species the average nucleotide distance was 0.054 ± 0.008 s.e. The lower range of the nucleotide distance when the standard estimate has been taken into consideration is 0.0046, and is above the point average estimate between *L. transtrifera* and *L. vanillana*. Similarly, the average nucleotide distance between the two *L. physophora* genetic groups (I & II; Fig. 3) was 0.103 ± 0.011 s.e., supporting the premise that taxonomic revision of the *Lobesia* genus is needed to better incorporate genetic and morphological data in this economically important moth genus.

Estimates of intra-specific uncorrected nucleotide distances (*P*-dist) with 500 bootstrap replications for standard error (s.e.) estimates involving all *L. botrana* unique mtDNA COI haplotypes(KX621012-KX621030, KX621032) suggested greatest nucleotide distances within *L.*

botrana were between the Italian haplotype IMEO16_17 and Israel haplotypes of ILMG01_10 and ILMG06_11 (0.011 ± 0.004 s.e.), between the Italian ITA1805 haplotype and Israel haplotypes ILMG01_10 and ILMG06_11, and between the Switzerland haplotype CHE1913_03 and Israel haplotypes ILMG01_10 and ILMG06_11.

Pairwise nucleotide distance (P-distance) of partial Cytb gene (577bp;GenBank accession numbers KX621033-KX621049, KX621051,KX621052) from *L. botrana* including standard errors (s.e.) estimated from 500 bootstrap replications. Pairwise nucleotide distances ranged from 0.002 ± 0.002 s.e.to 0.016 ± 0.005 s.e.were observed. The average nucleotide diversity between the 19 *L. botrana*Cytb haplotypes with the cryptic *L. botrana* haplotype (haplotype 20; KX621050) was 0.084 ± 0.010 s.e. (range 0.080 ± 0.011 s.e. to 0.088 ± 0.011 s.e.) (data not shown).

3.8 Concatenated mtDNA COI and Cytb haplotype network of Lobesia botrana

A haplotype network was constructed for *L. botrana* based on 1,277bp of concatenated partial mtDNA COI and Cytb gene sequences of all unique haplotypes against sampling localities (Fig. 5).Three haplotypes, (18,11), (02,11), and (02,01), were shown to have the highest frequencies in the *L. botrana* samples collected, and the number of SNPs between haplotypes ranged from one to six (e.g., between haploytpes (11,07) and (09,08)). Eighty-three SNPs separated between the potential cryptic/misidentified *L. botrana* (i.e., haplotype (20,20)) and its closest *L. botrana* neighbour (i.e., haplotypes (18,21) or (10,06). The haplotype network also showed that 21 of the 40 haplotypes identified in this study were present as singletons.

3.9 Population haplotype signatures

A *L. botrana* haplotype signature table for all sampled individuals at the population level was generated by considering both partial mtDNA COI and Cytb gene regions and is presented below (Table 5). A total of 21 mtDNA COI haplotypes were identified in this study, and this included one individual (FRA_23-10) that was distantly clustered with the majority EGVM mtDNA COI haplotypes. Similarly, the FRA_23-10 individual also possessed a very divergent Ctyb partial gene sequence from all sampled EGVM individuals. Including this FRA_23-10 individual (haplotype (20,20)), a total of 20 Ctyb haplotypes was detected in this study.

The global haplotype signature patterns indicated that within the New World invasive ranges of South America, three different mtDNA haplotypes were identified in Chile (i.e., (02,08), (02, 11), and (18,11)) based on the two-gene system developed here. If the Cytb marker was not included the number of matrilines in Chile would have been underestimated to only mtDNA COI haplotype 02 and haplotype 18. In the United States populations, two matrilines were also

identified (i.e., (18, 11), and (02, 22)), with the haplotype (18, 11) being shared between Chile and the United States.

Within individual countries representing potential traditional habitat range, eight *L. botrana* haplotypes (i.e., (01,14), (02,01), (02,11), (18,01), (18,11), (18,14), (19,11), and (21,11)) and one potential cryptic/misidentified*L. botrana* haplotype (i.e., (20,20)) were identified from a total of 151 French (FRA) collected individuals. A total of five haplotypes (i.e., (02, 09), (02,11), (03,12), (18,11), and (18,15)) were identified from 36 *L. botrana* individuals from Switzerland (CHE); four haplotypes (i.e., (02,11), (05,11), (18,08), and (18,11)) were detected from 30 *L. botrana* individuals from Germany (DKAI, DLOR), six haplotypes (i.e., (02,11), (06,08), (07,03), (07,08), (08,03), and (18,11)) were detected from 15 *L. botrana* from Spain (EYEL), 11 haplotypes (i.e., (02,02), (02,08), (02,10), (02,11), (02,15), (02,16), (03,12), (05,11), (14,08), (16,08), (17,11), and (18,11)) were detected from 42 *L. botrana* individuals from Italy (IMEO, ITA18, IVAL), and five haplotypes (i.e., (02,08), (15,11), (18,03), (18,11), and (18,21)) were identified from 37 *L. botrana* individuals from Portugal (PRT26, PRT27, PRT28, PRT29). Finally, from the eastern Mediterranean Basin regions, four haplotypes (i.e., 02,08), (02,11), (04,11), (05,17)) were detected from 29 EGVM from Turkey (TMMA, TMCA), eight haplotypes (i.e., (09,04), (09,05), ((09,08), ((10,06), (11,04), (11,07), (12,08), (13,08)) detected in 16 individuals from Israel (ILMG), and four haplotypes (i.e., (12,08), (13,08), (13,11), (13,13)) were identified from 15 *L. botrana* individuals from Syria (SYKA).

Table 8: Average uncorrected (*P*-dist) interspecific partial mtDNA COI (655bp) nucleotide distances for all *Lobesia* species reported in the iBoL and GenBank DNA databases, and included *L. botrana* unique haplotypes identified in the current study (KX621012 - KX621030, KX621032). The inter-specific estimates of average nucleotide distance between *Lobesia* species and the potential cryptic *L. botrana* species were calculated by combining the individual (FRA_23-10; KX621031) detected in this study, as well as two iBoL sequences (NLLEA1226-13, GWORB3724-14) from individuals collected from the Netherlands and Germany, respectively. Standard errors from 500 bootstrap replications are provided in blue (top triangle). Lowest and highest interspecific nucleotide distances are indicated in red.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*L. botrana*	-	0.008	0.009	0.009	0.007	0.009	0.009	0.008	0.008	0.010	0.010	0.010	0.011	0.011	0.012
2	*L. botrana* (cryptic)	0.054	-	0.010	0.010	0.008	0.010	0.010	0.009	0.010	0.011	0.010	0.011	0.012	0.012	0.013
3	*L. xylistis*	0.066	0.075	-	0.008	0.008	0.009	0.009	0.009	0.010	0.010	0.009	0.009	0.011	0.011	0.012
4	*L. semosa*	0.066	0.071	0.052	-	0.008	0.008	0.010	0.009	0.009	0.010	0.010	0.010	0.011	0.012	0.013
5	*L. vanillana*	0.049	0.063	0.051	0.053	-	0.007	0.008	0.008	0.008	0.009	0.010	0.010	0.011	0.011	0.012
6	*L. transtrifera*	0.066	0.066	0.062	0.053	0.041	-	0.009	0.009	0.009	0.009	0.010	0.010	0.011	0.011	0.012
7	*L.* sp. A	0.064	0.078	0.062	0.063	0.051	0.059	-	0.010	0.008	0.010	0.010	0.011	0.011	0.011	0.012
8	*L. reliquana*	0.049	0.056	0.069	0.069	0.059	0.069	0.076	-	0.009	0.010	0.011	0.010	0.012	0.011	0.013
9	*L.* sp. D	0.067	0.075	0.074	0.063	0.056	0.059	0.056	0.074	-	0.009	0.010	0.011	0.011	0.011	0.012
10	*L. physophora* (grp I)	0.077	0.098	0.078	0.076	0.067	0.066	0.076	0.085	0.069	-	0.011	0.013	0.012	0.012	0.013
11	*L. physophora* (grp II)	0.087	0.085	0.075	0.076	0.078	0.074	0.081	0.085	0.087	0.103	-	0.011	0.011	0.010	0.012
12	*L.* sp. (site=PNG)	0.086	0.080	0.068	0.069	0.073	0.073	0.093	0.088	0.092	0.105	0.099	-	0.012	0.011	0.012
13	*L. aresophanes*	0.112	0.118	0.090	0.092	0.098	0.098	0.093	0.117	0.102	0.118	0.090	0.111	-	0.011	0.012
14	*L. peltophora*	0.107	0.115	0.099	0.101	0.103	0.112	0.100	0.108	0.111	0.112	0.097	0.118	0.103	-	0.011
15	*L.* sp. ANIC 6	0.112	0.121	0.115	0.115	0.106	0.111	0.106	0.125	0.101	0.123	0.105	0.118	0.115	0.103	-

Table 9: Average uncorrected (*P*-dist) intraspecific partial mtDNA COI (700bp) nucleotide distances for all *Lobesiabotrana* unique haplotypes(KX621012-KX621030, KX621032). Standard errors from 500 bootstrap replications are provided in blue (top triangle). Lowest and highest intraspecific nucleotide distances are 0.001 ± 0.001 s.e.and 0.011± 0.004 s.e.and are detected between various haplotypes.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	COI_Hap01		0.002	0.002	0.002	0.002	0.003	0.003	0.003	0.003	0.004	0.004	0.002	0.003	0.002	0.002	0.002	0.002	0.001	0.002	0.002
2	COI_Hap02	0.003		0.001	0.001	0.001	0.003	0.003	0.003	0.003	0.003	0.004	0.002	0.002	0.001	0.002	0.002	0.002	0.001	0.002	0.002
3	COI_Hap03	0.004	0.001		0.002	0.002	0.004	0.003	0.004	0.003	0.004	0.004	0.003	0.002	0.002	0.002	0.003	0.003	0.002	0.002	0.002
4	COI_Hap04	0.004	0.001	0.003		0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.002	0.002	0.002	0.003	0.003	0.002	0.002	0.002
5	COI_Hap05	0.004	0.001	0.003	0.003		0.003	0.003	0.004	0.003	0.003	0.003	0.003	0.002	0.002	0.002	0.003	0.003	0.002	0.002	0.002
6	COI_Hap06	0.009	0.009	0.010	0.007	0.007		0.001	0.002	0.002	0.002	0.002	0.002	0.003	0.004	0.003	0.004	0.004	0.003	0.003	0.003
7	COI_Hap07	0.007	0.007	0.009	0.006	0.009	0.001		0.001	0.002	0.003	0.003	0.002	0.002	0.003	0.003	0.003	0.004	0.003	0.003	0.003
8	COI_Hap08	0.009	0.009	0.010	0.007	0.010	0.003	0.001		0.003	0.003	0.003	0.002	0.003	0.003	0.003	0.004	0.004	0.003	0.003	0.003
9	COI_Hap09	0.009	0.009	0.010	0.007	0.007	0.003	0.004	0.006		0.001	0.001	0.002	0.003	0.003	0.003	0.004	0.004	0.003	0.003	0.003
10	COI_Hap10	0.010	0.010	0.011	0.009	0.009	0.004	0.006	0.007	0.001		0.002	0.003	0.003	0.004	0.004	0.004	0.004	0.003	0.004	0.004
11	COI_Hap11	0.010	0.010	0.011	0.009	0.009	0.004	0.006	0.007	0.001	0.003		0.003	0.003	0.004	0.004	0.004	0.004	0.003	0.004	0.004
12	COI_Hap12	0.004	0.004	0.006	0.006	0.006	0.004	0.003	0.004	0.004	0.006	0.006		0.001	0.003	0.002	0.003	0.003	0.002	0.002	0.002
13	COI_Hap13	0.006	0.003	0.004	0.004	0.004	0.006	0.004	0.006	0.006	0.007	0.007	0.001		0.002	0.003	0.003	0.003	0.002	0.003	0.003
14	COI_Hap14	0.004	0.001	0.003	0.003	0.003	0.010	0.009	0.007	0.010	0.011	0.011	0.006	0.004		0.001	0.003	0.003	0.002	0.002	0.002
15	COI_Hap15	0.003	0.003	0.004	0.004	0.004	0.009	0.007	0.006	0.009	0.010	0.010	0.004	0.006	0.001		0.002	0.002	0.001	0.002	0.002
16	COI_Hap16	0.004	0.004	0.006	0.006	0.006	0.010	0.009	0.010	0.010	0.011	0.011	0.006	0.007	0.006	0.004		0.003	0.002	0.002	0.003
17	COI_Hap17	0.004	0.004	0.006	0.006	0.006	0.010	0.009	0.010	0.010	0.011	0.011	0.006	0.007	0.006	0.004	0.006		0.002	0.002	0.002
18	COI_Hap18	0.001	0.001	0.003	0.003	0.003	0.007	0.006	0.007	0.007	0.009	0.009	0.003	0.004	0.003	0.001	0.003	0.003		0.001	0.001
19	COI_Hap19	0.003	0.003	0.004	0.004	0.004	0.009	0.007	0.009	0.009	0.010	0.010	0.004	0.006	0.004	0.003	0.004	0.004	0.001		0.002
20	COI_Hap21	0.003	0.003	0.004	0.004	0.004	0.009	0.007	0.009	0.009	0.010	0.010	0.004	0.006	0.004	0.003	0.004	0.004	0.001	0.003	

Table 10: Average uncorrected (*P*-dist) intraspecific partial mtDNA Cytb (577bp) nucleotide distances for all *Lobesiabotrana* unique haplotypes(KX621033-KX621049; KX621051, KX621052). Standard errors from 500 bootstrap replications are provided in blue (top triangle). Lowest and highest intraspecific nucleotide distances are 0.002 ± 0.002 s.e.and 0.016 ± 0.005 s.e.and are detected between various haplotypes.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	Cytb_Hap01		0.002	0.002	0.004	0.003	0.004	0.003	0.003	0.003	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.003
2	Cytb_Hap02	0.002		0.002	0.004	0.004	0.004	0.004	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.003
3	Cytb_Hap03	0.003	0.002		0.004	0.004	0.004	0.003	0.002	0.002	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
4	Cytb_Hap04	0.009	0.010	0.009		0.002	0.002	0.002	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.004
5	Cytb_Hap05	0.007	0.009	0.010	0.002		0.002	0.002	0.004	0.005	0.004	0.004	0.005	0.004	0.004	0.004	0.004	0.004	0.005	0.004
6	Cytb_Hap06	0.009	0.010	0.012	0.003	0.002		0.003	0.005	0.005	0.004	0.004	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
7	Cytb_Hap07	0.007	0.009	0.007	0.002	0.003	0.005		0.004	0.004	0.004	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
8	Cytb_Hap08	0.005	0.003	0.002	0.010	0.012	0.014	0.009		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.002
9	Cytb_Hap09	0.007	0.005	0.003	0.012	0.014	0.016	0.010	0.002		0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
10	Cytb_Hap10	0.005	0.007	0.005	0.010	0.012	0.010	0.009	0.003	0.005		0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.003	0.002
11	Cytb_Hap11	0.003	0.005	0.003	0.009	0.010	0.012	0.007	0.002	0.003	0.002		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
12	Cytb_Hap12	0.005	0.007	0.005	0.010	0.012	0.014	0.009	0.003	0.005	0.003	0.002		0.002	0.002	0.002	0.003	0.002	0.003	0.002
13	Cytb_Hap13	0.005	0.007	0.005	0.010	0.012	0.014	0.009	0.003	0.005	0.003	0.002	0.003		0.002	0.002	0.002	0.002	0.003	0.002
14	Cytb_Hap14	0.005	0.007	0.005	0.010	0.012	0.014	0.009	0.003	0.005	0.003	0.002	0.003	0.003		0.002	0.002	0.002	0.003	0.002
15	Cytb_Hap15	0.005	0.007	0.005	0.010	0.012	0.014	0.009	0.003	0.005	0.003	0.002	0.003	0.003	0.003		0.002	0.002	0.003	0.002
16	Cytb_Hap16	0.005	0.007	0.005	0.010	0.012	0.014	0.009	0.003	0.005	0.003	0.002	0.003	0.003	0.003	0.003		0.002	0.003	0.002
17	Cytb_Hap17	0.005	0.007	0.005	0.010	0.012	0.014	0.009	0.003	0.005	0.003	0.002	0.003	0.003	0.003	0.003	0.003		0.003	0.002
18	Cytb_Hap21	0.007	0.009	0.007	0.012	0.014	0.016	0.010	0.005	0.007	0.005	0.003	0.005	0.005	0.005	0.005	0.005	0.005		0.003
19	Cytb_Hap22	0.005	0.007	0.005	0.010	0.012	0.014	0.009	0.003	0.005	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.005	

Figure 5:*Lobesia botrana* mtDNA haplotype network from partial concatenated mtDNA COI and Ctyb gene sequences (1,277bp). All *Lobesia botrana* haplotypes detected in this study differed from their closest neighbours by one to six nucleotide substitutions, with the exception of the FRA_23-10 individual from France (haplotype (20,20); see Table 11), and is 83 substitutions from its next closest *L. botrana* haplotypes of (10,06) or (18,21), lending support that this individual (FRA_23-10) belongs to a different species.

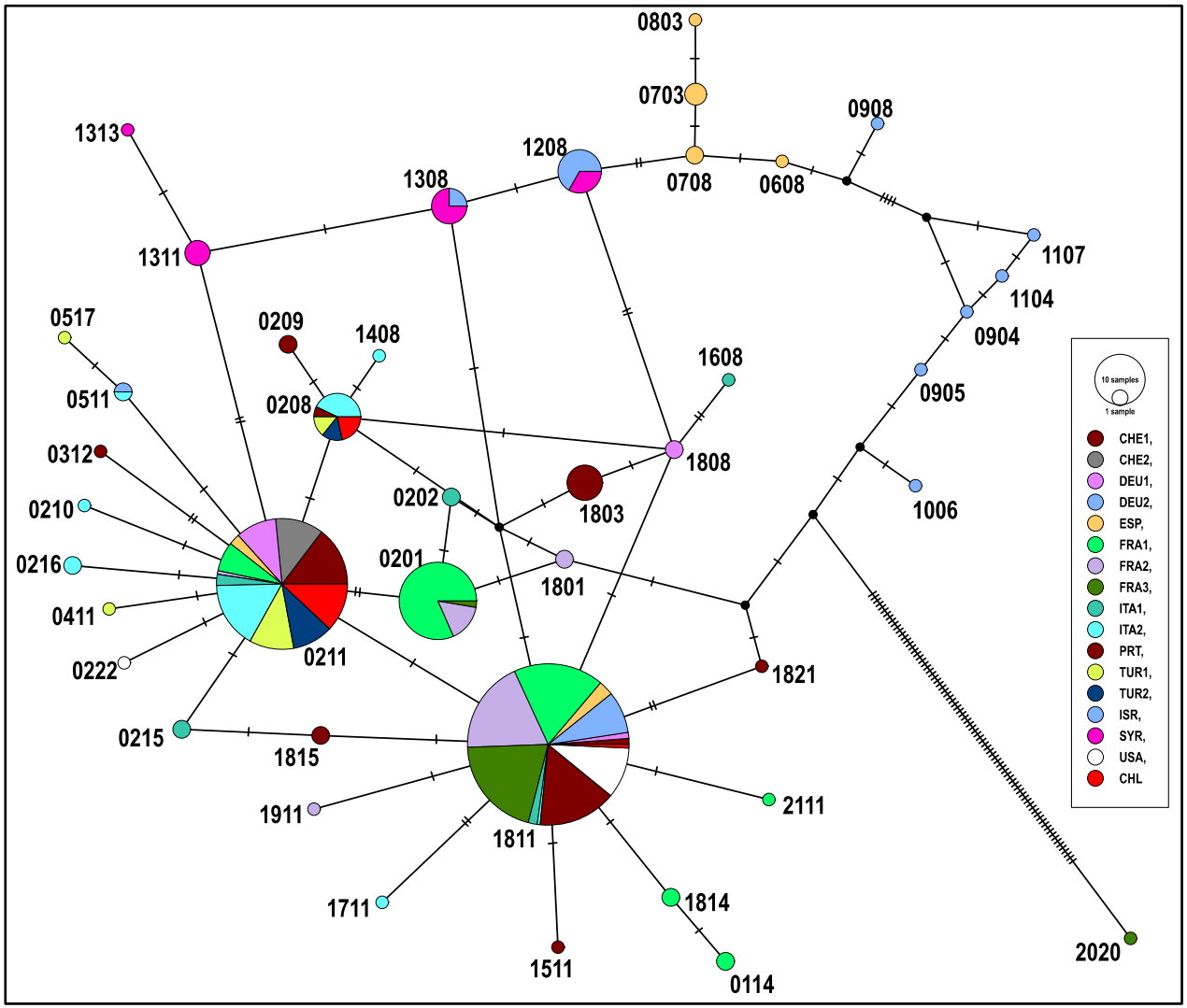


Table 11: Haplotype signatures of all *Lobesia botrana* individuals sampled from 37 populations representing 11 countries. Country codes are: CHE (Switzerland), FRA (France), EYEL(Spain), PRT (Portugal), DKAI and DLOR(Germany), ITA, IMEO, and IVAL (Italy), ILMG(Israel), TMMA and TMCA(Turkey), SYKA(Syria), CHL (Chile), and USA (United States of America; Laboratory population). Haplotype groups are clustered first by COI haplotype ID, followed by Ctyb haplotype ID (e.g., (01,01) represent COI haplotype 01 and Cytb haplotype 01). Numbers of individuals identified with particular haplotype signature are indicated according to sampling sites. Grouping of sampling sites are as indicated in Table 1.

haplotype	CHE19	CHE20	CHE30	CHL38-40	CHL41,43,44	CHL42,45	CHL46	DKAI	DLOR	EYEL	FRA04	FRA05	FRA06	FRA07	FRA08	FRA11	FRA12	FRA13	FRA14	FRA15	FRA16	FRA21	FRA22	FRA23	FRA31	IMEO	ITA18	IVAL	PRT26	PRT27	PRT28	PRT29	TMCA	TMMA	ILMG	SYKA	USA	total
1 (01,14)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
2 (02,01)	0	0	0	0	0	0	0	0	0	0	7	5	0	0	1	5	0	6	7	0	4	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	38
3 (02,02)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2
4 (02,08)	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	5	0	0	1	0	2	2	0	0	0	14
5 (02,09)	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
6 (02,10)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
7 (02,11)	5	11	13	3	6	3	1	11	0	3	0	2	0	0	0	0	1	2	0	3	1	0	0	0	0	10	3	8	0	0	0	0	12	11	0	0	0	109
8 (02,15)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2
9 (02,16)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2
10 (03,12)	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
11 (04,11)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
12 (05,11)	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2
13 (05,17)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
14 (06,08)	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
15 (07,03)	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
16 (07,08)	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
17 (08,03)	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
18 (09,04)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
19 (09,05)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
20 (09,08)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
21 (10,06)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
22 (11,04)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
23 (11,07)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
24 (12,08)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	4	0	12
25 (13,08)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	6	0	8
26 (13,11)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4
27 (13,13)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
28 (14,08)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
29 (15,11)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
30 (16,08)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
31 (17,11)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
32 (18,01)	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
33 (18,03)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	8
34 (18,08)	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
35 (18,11)	2	0	0	0	0	1	0	2	14	5	4	3	10	1	4	5	11	3	4	7	7	11	12	11	2	1	3	0	6	9	10	1	0	0	0	0	17	166
36 (18,14)	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
37 (18,15)	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
38 (18,21)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
39 (02,22)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
40 (19,11)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
41 (20,20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
42 (21,11)	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
total	12	11	13	3	6	5	3	15	15	15	12	11	10	2	6	11	12	12	11	12	12	11	13	12	4	16	11	15	10	13	13	1	16	13	16	15	18	406

3.10 *Lobesia botrana* rapid species identification via PCR-RFLP

In silico analysis of 18 partial mtDNA COI gene sequences of *Lobesia* species from the iBoL and GenBank public sequence databases, as well as from field individuals in this study, identified the restriction enzymes *Sml*I and *Dde*I as suitable for rapid *L. botrana* species differentiation. Enzyme recognition sites for these enzymes were present in the sequences from *L. botrana* but not those of other species. A PCR-RFLP-specific pair of primers (Appendix B) were optimised and amplicons digested by the *Sml*I and *Dde*I restriction enzymes confirmed that the two-step RFLP protocol was capable of distinguishing between *L. botrana* individuals (including the potential cryptic *L. botrana* species) and 17 other reported *Lobesia* species (Table 2).The PCR product from*L. botrana*candidates(including the *L. botrana* cryptic species) was cut once by the *Sml*I restriction enzyme (Fig. 6a), whilst the product from other *Lobesia* species was uncut. A restriction enzyme digest using *Dde*I then allows *L. botrana* to be differentiated from the cryptic *L. botrana*species (Fig 6b)through the presence of one restriction site in the cryptic species but absent in *L. botrana*.

Figure 6: (a) PCR-RFLP of *Lobesia botrana* (samples 1-3) and a potentially misidentified/cryptic species of *L. botrana* using the restriction enzyme *Sml*I. This first restriction digest step is designed to differentiate all possible *L. botrana* (one cut site generating two fragments of 364bp+291bp) from other known *Lobesia* species (no cut, 655bp expected). (b): This restriction digest step using the restriction enzyme *Dde*I was designed to differentiate *L. botrana* (no cut, 655bp) from the potential misidentified/cryptic *L. botrana* species (one cut site, generating two fragments of 405bp+250bp).

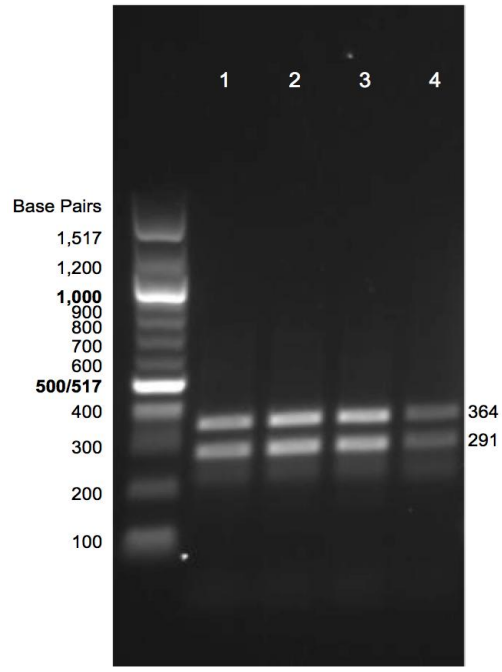


Fig.6a

655bp mtDNA COI partial gene digested by SmlI

Samples 1, 2, 3:*Lobesia botrana*

Sample 4:*Lobesia botrana* 'misidentified/cryptic'

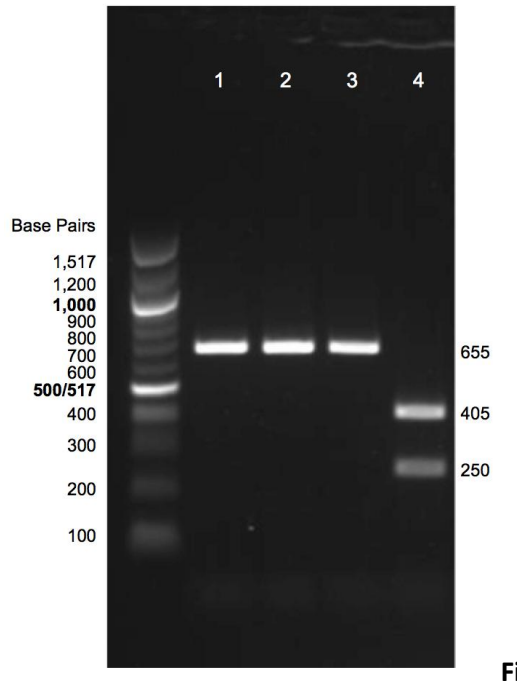


Fig. 6b

655bp mtDNA COI partial gene digested by *Dde*I

Samples 1, 2, 3:*Lobesia botrana*

Sample 4:*Lobesia botrana* 'misidentified/cryptic'

Part II Discussion

Identifying real *Lobesia botrana* mtDNA COI sequences

This study represents the most comprehensive population genetic survey carried out to date of the European grape vine moth (EGVM) *Lobesia botrana*. The survey covered its presumed Palearcticrange which includes the European and Asia/Central Asia regions (Fig. 1). Furthermore, this study also included survey of genetic diversity at the mtDNA genome in recent invasive populations from South (i.e., Chile) and North (i.e., USA) American continents.The analysis of population-wide genetic variability at the mtDNA COI gene region for *L. botrana*,together with interspecific comparisons, allowed identification of *L. botrana*-specific DNA sequences. This in turn allowed development of a simple PCR-RFLP method for species identification of *L. botrana*(Appendix B).In addition, knowledge of the extent of intraspecific nucleotide divergence within *L. botrana* allowed two divergent partial mtDNA COI haplotypes, reported in the iBoL database as being *L. botrana*, to be identified as being from a different species. Survey of *L. botrana* across its Palearctic range, as well as its invasive New World range identified 41 unique mtDNA haplotypes from >400 individuals, which all fell within the expected intra-species nucleotide distance range of between 0 and <2%, as is generally observed for intra-species comparisons (e.g., Behere et al. 2007; Tay et al. 2014; Tay et al. 2016), especially in insects.This therefore provided confident that a true EGVM mtDNA COI signature range has been identified.

Application of mtDNA database to understand incursion patterns

The history of *L. botrana* incursions into the New World indicated that the process first took place in South America (Chile), followed by detection in Argentina. Within one year of the South American incursions, *L. botrana* was also reported in the USA (Gilligan et al. 2011). It has not yet been ascertained what frequency of incursions took place in the Southern American countries of Chile and Argentina. Furthermore, no genetic data existed to link if incursions between Chile and Argentina were associated. Similarly, frequencies of incursion and genetic diversity in the North American populations of *L. botrana* were unknown, as was a possible association with South American invasive populations.The mtDNA database generated here (Table 2) can be used to trace the origins of the New World incursions. From the mtDNA dataset generated (Table 2), three matrilines were detected in Chile populations [(02,08) (02,11) and (18,11)], while in the US, two matrilines, (18,11) and (02,22), were detected. Introductions of EGVM into the South and North Americas therefore involved multiple matrilines, suggestive of multiple incursion events whereby each matriline represented a single incursion event. Because of the detection of the globally common (18,11) haplotype in both Chile and the USA, it is not possible to conclude if the USA incursion and the Chile incursion shared a common origin, nor was it possible to ascertain if the EGVM used Chile as a stepping stone into the USA. It is also possible that the multiple matrilines detected in both Chile and the USA were results of separate single

incursion events, whereby multiple femalefoundresses were introduced as a single event into either the USA or Chile (i.e., on a single contaminated consignment). From the intra-species ML phylogeny (Fig. 4), those matrilines that had successfully established in the New World were found in clades II, III and IV. Following this hypothesis the common country (i.e., Italy) shared between the three invasive lineages might indicate the country of origin for the New World populations. This study demonstrates the usefulness of the database and its potential as an Australian biosecurity toolkit for the table and wine grape industry (and for other horticultural industries that are potential target hosts for the EGVM).

Previously,Reineke et al. (2015) applied a suite of 10 microsatellite DNA markers to study gene flow patterns and population substructure in 14 EGVM populations from Turkey, Syria, Israel, Germany, Italy and Spain. The authors found significant heterozygote deficit from significant *F*is estimate, as well as moderate level of *F*st, suggested potential of inbreeding and reduced migration between Asian and European populations. The pattern is supported by the mtDNA where predominant 'Asia' (i.e., Clade I) and 'French' (I.e., Clade IV) clades were identified. Pairwise *F*st estimates from Reineke et al. (2015) indicated a general lack of gene flow between Asian and various European populations such as in Italy and Germany, although there was also substantial admixture between Asian and European populations. A cautionary note that relates to interpreting population substructure and gene flow patterns in the Lepidoptera based on microsatellite DNA markers is the likelihood ofmarker association with mobile elements (e.g., Tay et al. 2010). This aspect should be taken into consideration when interpreting the findings in*L. botrana*. The study of Reineke et al. (2015) also lacked samples representing the invasive range of*L. botrana*, as well as lacking populations from other major wine producing regions in the EU, including France and Portugal. With the dataset and population samples collected for this study, future opportunities exist to better understand the invasive pattern of EGVM from its native range into the New World through analysis of genome-wide single nucleotide polymorphisms (SNPs) to infer migration edges (S. Elfekinand P. De Barro, Pers. comm.) such as has been performed in the invasive global whitefly *Bemisia tabaci* MED and MEAM1 species complex (Elfekih et al. submitted).

Cryptic species in *Lobesia botrana*?

It is becoming increasingly common that cryptic species (i.e., individuals that are morphologically indistinguishable but genetically divergent), especially in insects, are being detected and reported based on molecular DNA markers. These included the global agricultural hemipteran pest *Bemisiatabaci* cryptic species complex (De Barro et al. 2011), the stored grain pest beetle *Cryptolestes*species (Tay et al. 2014), and various lepidopteran pests (e.g., De Souza et al. 2015). In the *Lobesia* genus, evidence of potential cryptic species and/or species misidentification has been identified in this study. Previously, in the iBoL mtDNA COI database four sequences identified as belonging to *L. botrana* were publicly available. These four sequences could be divided into two genetically divergent groups (two from Greece/Crete; and one each from Netherland and Germany; Table 2) with*ca*. 5.4% nucleotide distancebetween them (Table 8).Inference of ML phylogeny, based on the partial mtDNA COI gene, clustered the Greece (Crete)

individuals with the majority of field-collected *L. botrana* specimens analysed in this study with a high bootstrap value (96.6%). Similarly, the Netherlands and Germany individuals identified as '*L. botrana*' within the iBoL database also clustered (100%) with one French *L. botrana* individual (FRA_23-10; COI region: KX621031, mitogenome: KX621053). However, these three *'L. botrana*' individuals were phylogenetically more closely related to *L. virulenta* (KP150241) (79.8%) than with the majority of the field-collected *L. botrana*, with these *L. virulenta*/'cryptic' *L. botrana* clades being placed as a sister clade to the *L. botrana* clade with a bootstrap confidence value of 59.4%. The presence of two phylogenetic clades both with the '*L. botrana*' species name raised the issue of identity of the real '*L. botran*a', and therefore also the true 'EGVM'. Although the probability of misidentification for the majority of specimens collected from vineyards for this study, as well as the specimens used in Reineke et al. (2015) is low, it is nevertheless necessary to reconfirm species status for *L. botrana* based on morphological characters, although the typed specimens of Denis &Schiffermüllerheld at the Austria museum had been lost (P Hebert, Pers. Comm.).

Possible misidentification and/or the presence of yet a second cryptic species in the *Lobesia* genus is further evident from the interspecific phylogenetic tree based on the partial mtDNA COI gene (Fig. 3). The species listed in Table 2 as *L. physophora* from Australia exhibited significant nucleotide differences in two groups: (i) HM372970 and KF399033, and (ii) GU688797, KF397870, GU688768, and KF402127. These two groups were phylogenetically divergent from each other, with mean nucleotide distance of > 5%. Other issues relating to phylogenetic placement of *Lobesia* species are further evident from this study, such as between *L. aeolepa*(KF523801) and *L. transtrifera* (GU688763, KF404301), and between the two *L. peltophora*sequences (GU688769, KF395899). Confidence in a species' phylogeny and taxonomic status cannot be underestimated and will underpin success of future pest management strategies. The aspects of cryptic species status and phylogenetic relationships of *Lobesia* species can be addressed using genome-wide SNP data and by applying robust phylogenetic analyses methodologies rather than by applying single or limited multi-gene analyses (S. Elfekih and P. De Barro, Pers. comm.).

RFLP for*Lobesia botrana* species identification

This study also provided the opportunity to develop a PCR-RFLP method for rapid distinctionbetween 17 *Lobesia* species, a cryptic/misidentified *L. botrana* species, and the true*L. botrana*. The protocol developed called for two enzyme digestion steps, however as other*Lobesia* species have yet to be characterised for their mtDNA COI gene, this PCR-RFLP protocol should be utilised with caution. Samples which include a large number of species and individuals mayinclude as yet uncharacterised examples, compromising the RFLP patterns and potentially leading to misidentification. At this stage, sequencing of suspect individuals based on the primers developed in this study (Appendix B) to enable direct nucleotide comparison with all *L. botrana* partial mtDNA COIsequences (GenBank accession numbersKX621012 - KX621032) is mandatory.

Appendix A Preservation protocol for field-collected *Lobesia botrana* specimens

Storage of field-collected Lepidopteran samples for molecular DNA purpose

This protocol is developed to enable short-term preservation of lepidopteran biological materials (adult moths, larvae) obtained from field-sites. The procedures involved are relatively straight-forward and requires the insects to be 'pickled' in high concentration of ethanol at low temperature over a period of 5-10 days. The concept is simple – by 'cold' pickling the insect in sufficient ethanol and over a sufficiently long period of time, this will ensure that the fluid in the insect is removed as much as possible in an environment (i.e., in a freezer) that will sustain the maximal integrity of the insect's DNA.



Fig. A. 50mL graduated Nunc tube



Fig. B. 15mL graduated Nunc tube

Instructions

[1.] put approximately 20-30 live insects in a sample tube (e.g., a 50mL volume Nunc tube, Fig. A) and top up with absolute ethanol (99.9%) and place in -18˚C to -20˚C (i.e., in a freezer) for 16-24 hours. For smaller sample size such as ≤ 10 individuals (adult moths/caterpillars) the insects can be placed in a small (e.g., 15mL volume) sample tube (Fig. B).

[2.] after the 16-24 hours, discard the used ethanol and top up with fresh absolute ethanol and return to the freezer for another 16-24 hours.

[3.] repeat step [2] for an additional 2 to 3 times on a daily basis, ensuring that this preservation step is done at between -18˚C to -20˚C.

[4.] once the ethanol preserving the insects have been refreshed daily over the 4-7 day period, the specimens can be posted / transported to a laboratory at room temperature.

[5.] once transported to a laboratory, ensure that the ethanol is replaced with fresh (≥95%) ethanol, and keep at -18˚C to -20˚C until ready for posting.

[6.] for sending the insects by air transport, please ensure that: (i) the sending institution has a CITES registration ID (or to register the sending institution with the Australian Government Department of the Environment), (ii) packaging of the insects in ethanol adheres to the IATA Dangerous Goods instructions, and (iii) that a copy of the CSIRO-provided letter accompanies each individual package of preserved samples.

Note 1: where possible, please use only live specimens (adult moths, pupae, larvae, eggs).

Note 2: if the insects can not be kept at -18˚C to -20˚C after being collected in the field, an alternative is to leave them in generous amount of ethanol at ambient temperature, but ensure that the ethanol is changed regularly, especially during the first 48 hrs (changing the ethanol every 6-8 hrs for the 24-48hrs).

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Appendix B Draft Diagnostic Protocol for EGVM molecular identification

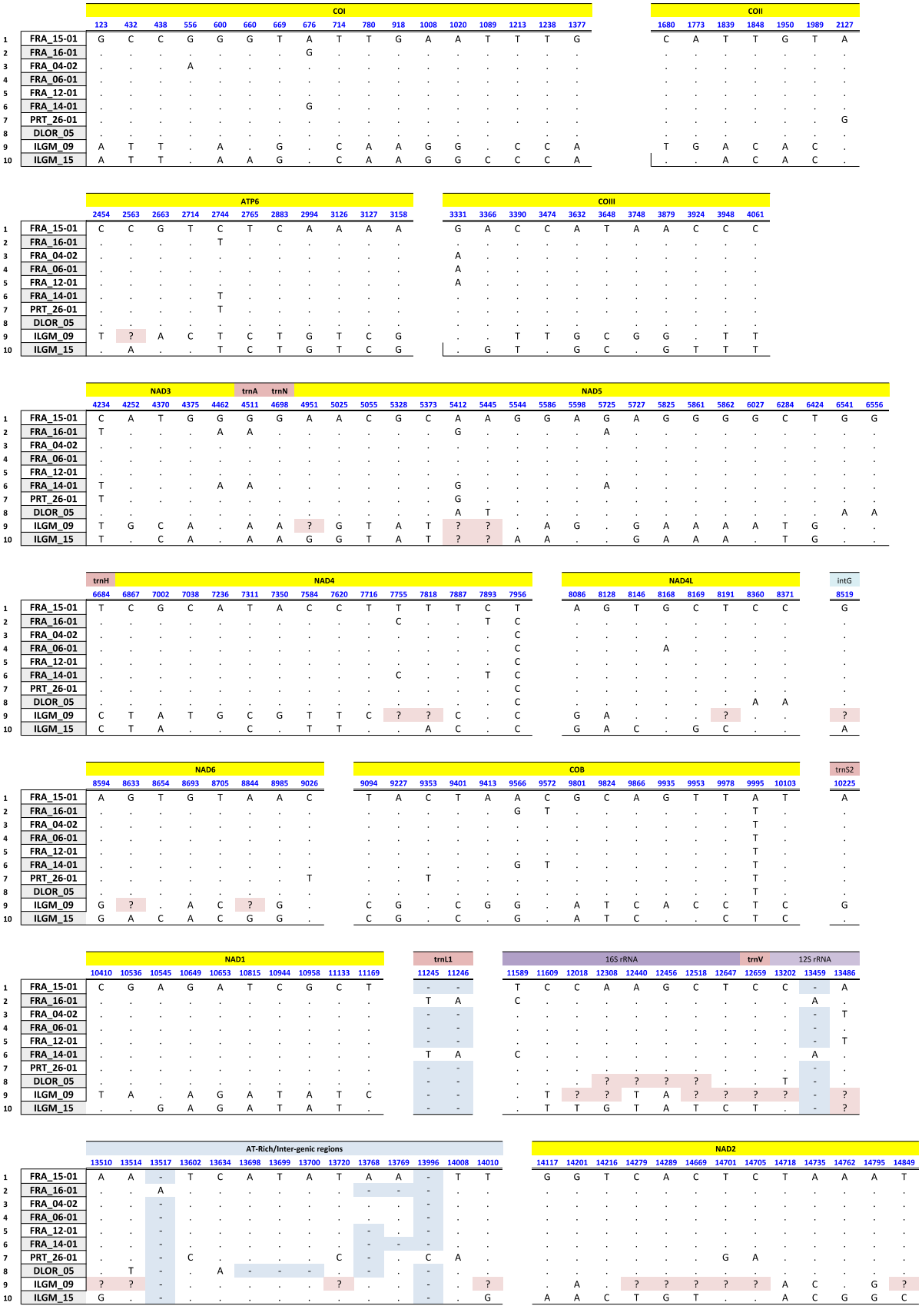
Note: Complete version of the Draft Diagnostic Protocol for EGVM is provided as Supplemental Material I

Draft Diagnostic Protocol for Molecular Identification of the European Grapevine Moth *Lobesia botrana* [Denis & Schiffermüller] (Lepidoptera: Tortricidae)



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Appendix C Summary of EVGM mitogenome SNPs and INDELs



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