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RESEARCH

Molecular characterization and first report of *Wolbachia* strains of the European grapevine moth *Lobesia botrana* (Lepidoptera: Tortricidae) in populations from Argentina

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Abstract The grapevine moth *Lobesia botrana* is a major pest of vineyards worldwide. Chemical insecticides and the sexual confusion technique are used to reduce damage. Several biological control approaches are being studied, including the use of the obligate intracellular bacteria *Wolbachia*, however, this method has been little explored. To use this bacterium for pest control it is necessary to know if the target pest is infected. The aim of this study was to examine *Wolbachia* infection in *L. botrana* populations from different vine growing areas of San Juan, Argentina. *Lobesia botrana* were captured in vineyards using sticky traps with pheromones. *Wolbachia* infection was diagnosed using a specific PCR test and fully characterized using Multi-Locus Sequence Typing (MLST) analyses. All populations tested were positive for *Wolbachia*, representing the first record of *Wolbachia* strains in grapevine moths from Argentina. The MLST analyses showed that *Wolbachia* strain belongs to supergroup B, clustering with other *Wolbachia* moth strains. More research is needed to understand the relationship between the grapevine moth and *Wolbachia* and how to use this to manage pests.

Keywords Biological control · *Wolbachia* strains · Molecular characterization · Manage pests · Grapevine moth

Introduction

The European grapevine moth, *Lobesia botrana* (Denis & Schiffermüller, 1775) (Lepidoptera: Tortricidae), is a polyphagous insect that can complete its life cycle using different host species (Ioriatti et al., 2011; Thiéry & Moreau, 2005). However, it is considered an agricultural pest due to its preference

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for Vitaceae as a food source (Thiéry & Desneux, 2018). Grapevine crops suffer significant losses due to larvae that feed on both floral buds and fruit, promoting the proliferation of phytopathogenic fungi and other organisms that lead to fruit loss (Fermaud & Le Menn, 1992; Roehrich & Boller, 1991). This pest is considered one of the most important in vineyards worldwide (Vicente-Díez et al., 2021).

Lobesia botrana was first recognized in 1800 in Austria. Subsequently, it gradually spread across Europe during the 19th and 20th centuries (Torres-Vila et al., 1995). In 2009, *L. botrana*, was detected in the western United States (Simmons et al., 2021; Varela et al., 2010). In South America, was detected in Chile in 2008, and later its presence was confirmed in the Mendoza province of Argentina in 2010. Since then, it has spread throughout the country and has been detected in the provinces of San Juan, Entre Ríos, and Salta (Benelli et al., 2023a; González, 2010; Ioriatti et al., 2012). In 2010, the National Service of Agri-Food Health and Quality (SENASA), a governmental organization that is responsible for executing the national policies regarding animal and plant quality and safety, declared a phytosanitary emergency due to the significant impact of this pest in Argentina (SENASA, 2010). Currently, *L. botrana* is present in the main wine-producing areas of Argentina (Taret et al., 2021).

National Programs are currently using chemical insecticides, biological products, and the mating disruption technique to control this pest (Taret et al., 2021). Recently promising pilot trials were conducted in Chile on the use of the Sterile Insect Technique (SIT) (Simmons et al., 2021). Several studies have been conducted to address various issues related to *L. botrana*, including the impact of irradiation on mating biology and behavior, mass rearing methods, field releases, and the evaluation of sterile moth competitiveness, longevity, and dispersal (Benelli et al., 2023a).

Additionally, various biological control strategies are being explored to combat this pest, including the use of predators such as insects, other arthropods, vertebrates (Coscollá, 1996; Ioriatti et al., 2011; Thiéry & Desneux, 2018), microorganisms such as entomopathogenic fungi and *Bacillus thuringiensis* (Beris et al., 2024; Vicente-Díez et al., 2021), and entomopathogenic nematodes (Campos-Herrera et al., 2023).

In this context, the potential of the endosymbiotic bacteria *Wolbachia* as a control agent in *L. botrana* has been scarcely explored. *Wolbachia* induces various reproductive alterations in its hosts, such as parthenogenesis, male killing, feminization, and cytoplasmic incompatibility (CI). CI is proposed as a strategy similar to the SIT for controlling insect pests (Werren et al., 2008) where males carrying a *Wolbachia* strain not present in the wild population, induce sterility. Recently, it has been discovered that *Wolbachia* may affect the susceptibility of its hosts to entomopathogenic bacteria (Díaz-Nieto et al., 2021) and pesticides (Li et al., 2018; Liu et al., 2024; Liu & Guo, 2019). It is important to note that there have been reports of resistance to certain insecticides in native populations of *L. botrana* in Italy, Turkey, and Greece (Benelli et al., 2023b). Therefore, it is essential to study the potential implications of *Wolbachia* in these mechanisms to propose appropriate control program strategies.

Wolbachia is present in approximately 50% of arthropods and has been found in various insects, mites, spiders, isopods, and nematodes (Kaur et al., 2021). *Wolbachia* infection is prevalent in lepidopterans, with reported infection frequencies of up to 80 (Ahmed et al., 2015; Duplouy & Hornett, 2018). A study on *Homona magnanima* Diakonoff, 1984 (Lepidoptera: Tortricidae) detected multiple infections with three different strains (Arai et al., 2019). Among the various phenotypes induced by *Wolbachia*, only cytoplasmic incompatibility and male killing have been reported in Tortricidae (Arai et al., 2019; Duplouy & Hornett, 2018). For *L. botrana*, the first and only report of *Wolbachia* infection has been in native populations of Portugal (Pazian et al., 2020), without a description of its effects on the host.

Identifying a particular strain of *Wolbachia* may offer insights into potential phenotypes induced by this bacterium in its host (Baldo et al., 2006). However, definitive confirmation of the effect exerted by the symbiont on its host requires conducting various biological assays.

This study presents a molecular characterization of *Wolbachia* strains found in native populations of *L. botrana* from a region in Argentina. The investigation on the *Wolbachia* infection status establishes a foundation for future biological research on the effects of

Wolbachia in populations of *L. botrana*. This research may lead to the proposal of biorational control strategies to be incorporated into management programs.

Material and method

Study area

Lobesia botrana male adults were collected from vineyards in San Juan province, Argentina (Fig. 1). The area has a continental-desert climate, with significant annual variations in temperature and pressure. Precipitation is scarce, primarily occurring during the summer months (December to March), with an average annual rainfall of 110 mm. The average annual temperature is 17.2 °C, and winter brings frost, with temperatures dropping as low as −8 °C (Cuesta et al., 2020). Although the province has a desert climate, an irrigation system provided by artificial canals, known as "acequias" or "ditches", supplies water to a variety of fruits and vegetables produced in the fertile valleys, as well as to urban trees, transforming rural and urban areas into a kind of oasis (Schliserman et al., 2014).

Insects and DNA extraction

To determine the prevalence of *Wolbachia* infection in wild *L. botrana* populations in the province of

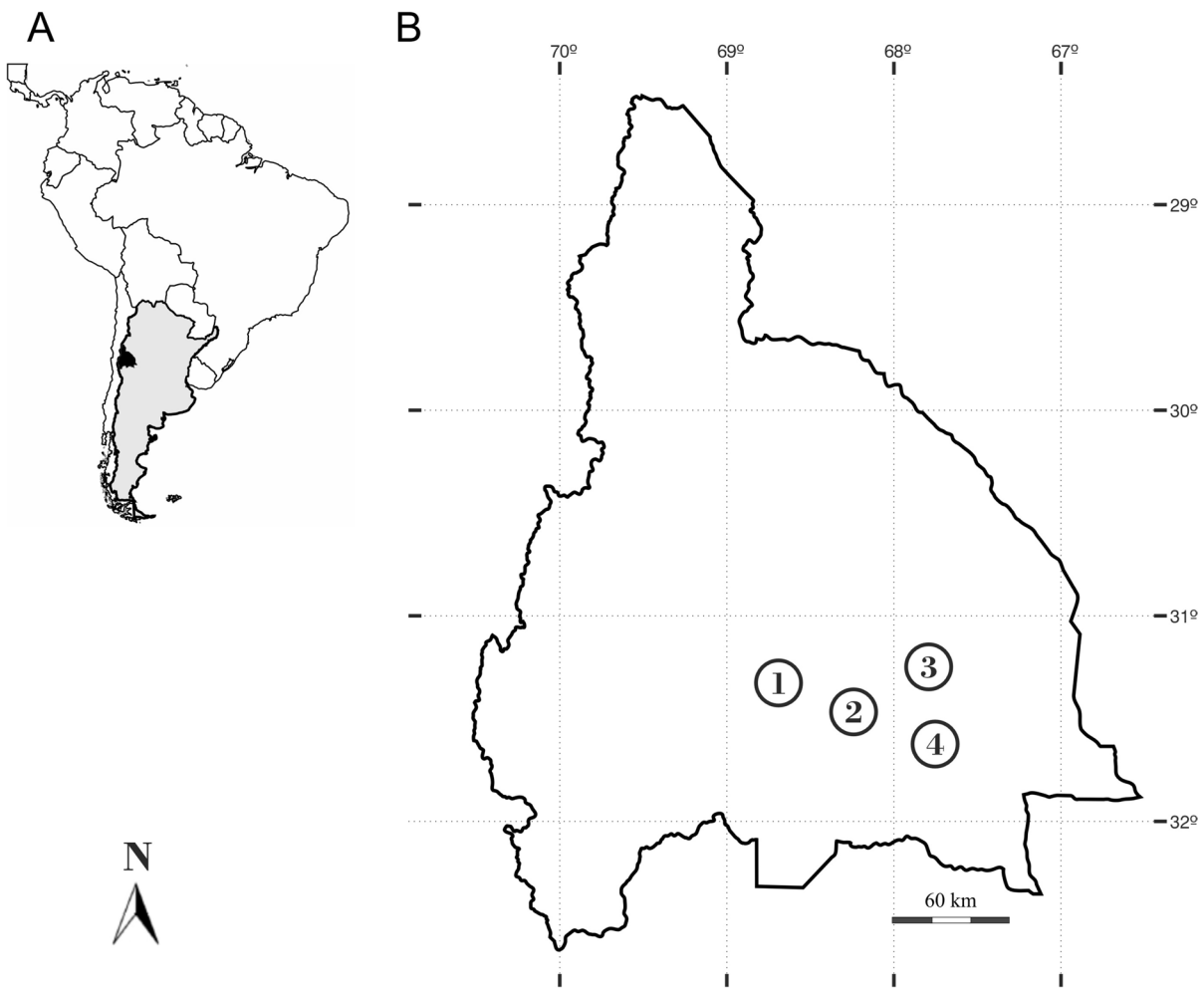


Fig. 1 Study area and detail of the location of the sampling points. **A** Location of San Juan in South America. **B** San Juan province, map shows the locations of the four sampling points (1: Ullum, 2: El Rincón, 3: Las Chacritas, 4: Puntilla)

San Juan, adult male specimens were collected from four vineyards during February-March 2023 (Fig. 1). Population sampling was done using commercial traps with specific pheromones (BioLure®). Fifteen traps were collected for each crop, and a total of 20 *L. botrana* individuals per crop were morphologically identified according to taxonomic keys (Gilligan et al., 2010) and individually preserved in Eppendorf tubes at −20 °C. The DNA extraction was carried out for each specimen separately using the PureLink Genomic DNA Mini Kit (Invitrogen, Grand Island, New York, USA), following manufacturer's instructions. The extraction products were stored at −20 °C for subsequent analysis. To verify the quality of DNA, the amplification of the cytochrome *c* oxidase (COI) gene was used according to Folmer et al. (1994).

Wolbachia detection

PCR was used to detect *Wolbachia* using the *wsp*-specific primers *wsp*−81 F and *wsp*−691R (Braig et al., 1998) from DNA extracted from single male moths (twenty per vineyard) as a template. PCR products were run on 1% agarose gel-electrophoresis and analyzed using a gel imaging system (SmartView Pro 1100 Imager System, UVCI-1100, USA). PCR-amplified DNA fragments were sequenced by an external service, Macrogen® (Korea).

Sequence analysis and molecular characterization of *Wolbachia* strains

Wolbachia infections were characterized through analysis of different *Wolbachia* markers: (1) the *Wolbachia* surface protein gene (*wsp*) (Table S1) according to Braig et al. (1998); (2) and trough detection of five house-keeping genes developed for the *Wolbachia* multilocus strain typing (MLST) methodology (primers: *gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*) as described by Baldo et al. (2006). For all PCR reactions described the appropriate negative (no DNA) and positive controls were included. A genomic DNA of *Anastrepha fraterculus* (Wiedemann, 1830) carrying *Wolbachia* was used as a positive control. The absence of *Wolbachia* was confirmed through the repetition of the test on two further occasions.

The PCR products were analyzed and sequenced as previously described. All sequences were manually edited, analyzed using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 11 (Tamura et al., 2021).

The distance between the *wsp* consensus sequences was calculated on both the nucleotide and amino acid levels using MEGA version 11 (Tamura et al., 2021). All gap-containing codons were deleted and homogeneous patterns between lineages and uniform rates between sites were assumed. Maximum composite likelihood was applied for nucleotides and Poisson correction for amino acids.

To build the phylogenies, we performed multiple alignments using the concatenated sequences of the genes (*gat*B, *cox*A, *hcp*A, *fts*Z, and *fbp*A) downloaded from https://pubmlst.org/wolbachia/ and detailed in Table S2. We included members from several *Wolbachia* supergroups. Phylogenetic relationships were analyzed using Maximum Likelihood (ML) methods in MEGA 11. The optimal evolutionary model was selected by critically evaluating the selected parameters, and the Find Best-Fit Substitution Model was conducted in MEGA 11 (Tamura et al., 2021). Bootstrap analysis was conducted with 1000 replications, and bootstrap values were calculated using a 50% majority rule.

Nucleotide sequence accession numbers

All newly generated sequences for *wsp*, *gatB*, *coxA*, *hcpA*, *ftsZ*, *fbpA* genes were deposited in the GenBank database under accession numbers: PP946296, PP946297, PP946298, PP946299 (*wsp* gen from hosts at the four sampling sites) and PP964735 (*gat*B), PP964737 (*coxA*), PP964736 (*hcp*A), PP964734 (*fts*Z), PP964733 (*fbp*A) respectively.

Results

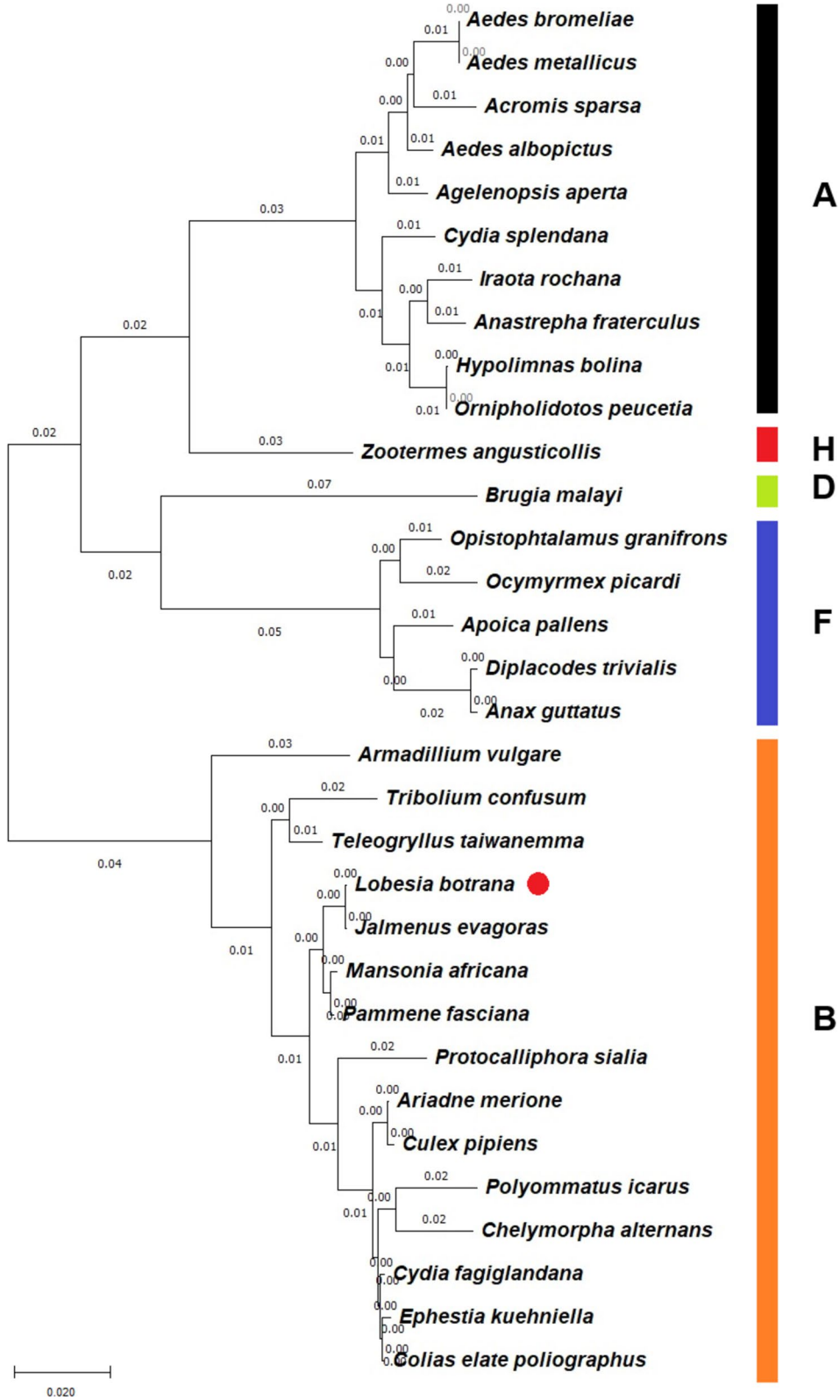
Wolbachia was present in all tested populations, although not all individuals per site were infected. The prevalence varied among locations: 100% in Las Chacritas (20/20), 70% in Puntilla (14/20), 85% in Ullum (17/20), and 80% in El Rincón (16/20).

Nucleotide and amino acid analysis based on the *wsp*-gen (Genbank Accession number: PP946296, PP946297, PP946298, PP946299) indicates that strains from four San Juan sites are identical to each other and to those from Portugal. Some differences

Table 1 Pairwise distances between *wsp* consensus sequences from different Lepidoptera families calculated at the nucleotide and amino acid levels[a]

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*Lobesia botrana* Las Chacritas SJ		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.329	0.154	0.308
2	*Lobesia botrana* Puntilla SJ	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.329	0.154	0.308
3	*Lobesia botrana* El Rincón SJ	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.329	0.154	0.308
4	*Lobesia botrana* Ullm SJ	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.329	0.154	0.308
5	*Lobesia botrana* clone 1 Portugal	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.314	0.184	0.303
6	*Lobesia botrana* clone 2 Portugal	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.314	0.184	0.303
7	*Lobesia botrana* clone 3 Portugal	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.314	0.184	0.303
8	*Phalera bucephala* (Notodontidae)	0.002	0.002	0.002	0.002	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.019	0.000	0.329	0.154	0.308
9	*Polyommatus icarus* (Lycaenidae)	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.019	0.000	0.329	0.154	0.308
10	*Erynnis tages* (Hesperiidae)	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.019	0.000	0.329	0.154	0.308
11	*Colias croceus* (Pieridae)	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.019	0.000	0.329	0.154	0.308
12	*Catoptria pinella* (Crambidae)	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.019	0.000	0.329	0.154	0.308
13	*Pammene fasciana* (Tortricidae)	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.007	0.007	0.007	0.007	0.007		0.020	0.329	0.169	0.329
14	*Sesamia inferens* (Noctuidae)	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.007		0.329	0.152	0.308
15	*Ariadne merione* (Nymphalidae)	0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.185	0.182		0.372	0.197
16	*Dendrolimus spectabilis* (Lasiocampidae)	0.091	0.091	0.091	0.091	0.104	0.104	0.104	0.089	0.089	0.089	0.089	0.089	0.094	0.087	0.233		0.372
17	*Papilio demoleus* (Papilionidae)	0.221	0.221	0.221	0.221	0.208	0.208	0.208	0.221	0.221	0.221	0.221	0.221	0.227	0.221	0.106	0.256	

[a]Distance for nucleotides is given below the diagonal and the distance for amino acids is given above the diagonal


Aedes bromeliae
Aedes metallicus
Acromis sparsa
Aedes albopictus
Agelenopsis aperta
Cydia splendana
Iraota rochana
Anastrepha fraterculus
Hypolimnas bolina
Ornipholidotos peucetia
Zootermes angusticollis
Brugia malayi
Opistophtalamus granifrons
Ocymyrmex picardi
Apoica pallens
Diplacodes trivialis
Anax guttatus
Armadillium vulgare
Tribolium confusum
Teleogryllus taiwanemma
Lobesia botrana
Jalmenus evagoras
Mansonia africana
Pammene fasciana
Protocalliphora sialia
Ariadne merione
Culex pipiens
Polyommatus icarus
Chelymorpha alternans
Cydia fagiglandana
Ephestia kuehniella
Colias elate poliographus
A
H
D
F
B
0.020

◂**Fig. 2** Maximum-likelihood tree constructed in MEGA 11 based on the concatenated MLST data of *Wolbachia* strains. Numbers indicate branch lengths. Colors and letters indicate different supergroups. The red dots on the graph indicate the *Wolbachia* strains of *L. botrana* that were sampled in the present study. Scale bar indicates branch lengths of the tree

were observed when comparing *L. botrana* sequences with those of other Lepidoptera species. Lowest pairwise distances were observed for *L. botrana* and *Pammene fasciana* (Tortricidae) on both nucleotide (0.008) and amino acid (0.019) levels. The highest pairwise genetic distance of *wsp* sequences was 0.221 (nucleotides) between *L. botrana* and *Papilio demoleus* (Papilionidae) and 0.329 (amino acids) between *L. botrana* and *Ariadne merione* (Nymphalidae). All distances are given in Table 1.

The MLST analyses revealed that the *Wolbachia* strain of *L. botrana* from San Juan belongs to supergroup B, clustering with isolated strains of the Hemiptera *Bemnisia tabaci* and separate from strains isolated from the tortricid *Cydia fagiglandana* and other supergroup B strains (Fig. 2).

Discussion

Understanding the current status of *L. botrana* populations regarding *Wolbachia* infection will be crucial for proposing strategies aligned with the needs of control programs. In this study, we report for the second time populations of *L. botrana* carrying *Wolbachia*, representing the first report for the southernmost region of the American continent.

The incidence of *Wolbachia* in Lepidoptera is high compared to the rest of the arthropods. It is estimated that approximately 80% of the Lepidoptera species tested are carriers of this bacterium (Ahmed et al., 2015). Pazian et al. (2020) detected 85% of the *L. botrana* individuals studied in Portugal were infected with *Wolbachia*. The percentage of infection in the populations analyzed in our work ranged from 70 to 100% demonstrating a high prevalence of *Wolbachia*. However, these percentages could be affected by false negatives that could be due to low titers of the bacterium or certain inhibitors in arthropod DNA (Jeyaprakash & Hoy, 2000).

Infection frequency varies according to transmission efficiency (Narita et al., 2009). Some Lepidoptera species exhibit extreme variations in *Wolbachia* prevalence (from 50 to 100%, from 0 to 40%) (Charlat et al., 2005; Delgado & Cook, 2009; Dyson & Hurst, 2004). Our work detected a high percentage of *Wolbachia*-infected individuals, and all populations analyzed were positive for the bacterium. This could suggest the presence of strains with high transmission efficiency and rapid dispersal among native populations.

Ahmed et al. (2015) presented evidence that climate and geography are strong predictors of the frequency of *Wolbachia* infection in Lepidoptera. The authors demonstrated that the level of infection varies with geographic distribution, following a latitudinal gradient. The lowest frequency is found at high latitudes, suggesting that infection rates are higher in warmer climates. Our results show that *Wolbachia* is highly prevalent in populations from an arid desert region with extreme temperatures. This region typically experiences very low temperatures during the winter (Cuesta et al., 2020). Thus, we report a high frequency of *Wolbachia* in regions with contrasting climates.

The analysis of the *wsp* gene sequences revealed a high degree of similarity among the different populations in our study area, as well as with populations from Portugal (Table 1). The fact that *L. botrana* populations in South America have similar strains to those in Europe could indicate a common origin and introduction of European populations into South America, probably entering from Chile to Argentina (Ioriatti et al., 2012). However, further population analyses could clarify this hypothesis.

The MSLT technique is an effective tool for detecting diversity among *Wolbachia* strains from the same host and identifying closely related strains from different arthropod hosts. This allows for investigation into the biology and evolution of this bacterium (Baldo et al., 2006). *Wolbachia* can be classified into 17 phylogenetic supergroups (A–F, H–Q, and S), with the majority of strains belonging to supergroups A and B. Some supergroups are only represented by a single strain (Kaur et al., 2021).

Wolbachia strains reported in Lepidoptera have corresponded to supergroups A, B, and F (Duplouy & Hornett, 2018), while in Tortricidae, only

supergroups A and B have been reported (Avtzis et al., 2014). This study presents the first MLST analysis for *L. botrana*, indicating that the strain found in the populations studied in this work belongs to supergroup B. A study on *Cydia fagiglandana* (Lepidoptera: Tortricidae) revealed that the strain present in this moth corresponds to supergroup B which exhibits a high prevalence of *Wolbachia* (77%) in the analyzed individuals (Avtzis et al., 2014). This result aligns with our findings, where the average prevalence was 84%. *Wolbachia* can manipulate the reproductive biology of Lepidoptera through various pathways (Duplouy & Hornett, 2018). However, the effects of *Wolbachia* on *L. botrana* have not yet been determined. Insects infected with supergroup B exhibit cytoplasmic incompatibility, male killing, and feminization (Werren et al., 2008). It is possible that these phenotypes may also be present in the native populations of Argentina studied in this work.

Detecting incompatibility strains in *L. botrana* would be of interest to propose control strategies for this pest. Conversely, some current control techniques such as the mating disruption technique (Gaston et al., 1967) would be rendered ineffective by strains that produce only female offspring. However, given our results, studies on closely related Lepidoptera, and the development of mass rearing for *L. botrana*, microinjection of mass reared males with *Wolbachia* strains not present in wild populations could result in the use of the Incompatible Insect Technique (IIT) for population suppression (Kittayapong et al., 2019).

It has been determined that certain strains of *Wolbachia* in insects can reduce their susceptibility to insecticides (Díaz-Nieto et al., 2021; Li et al., 2018; Liu et al., 2012). The presence of *Wolbachia* in the various populations analyzed, along with the rapid expansion of *L. botrana* in the region, despite control efforts that include the application of insecticides (Taret et al., 2021), suggests that this endosymbiont may confer certain adaptive advantages to carrier populations. However, this will have to be determined in future trials. It is also possible that the presence of invasive linages may be a contributing factor (Men et al., 2013). Furthermore, the use of insecticides may act as an agent of selection, resulting in the emergence of resistant genotypes of this moth (Katsavou et al., 2023).

Additional research is required to fully explore the relationship between the grapevine moth and *Wolbachia*. This will enable the development of pest management strategies based on this symbiotic relationship. To establish the relationship between strains from different regions, more sequences need to be added to databases. Finally, our work is a necessary first step in developing sustainable environmentally-friendly pest management techniques.

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Authors' contributions L.M.D.N. and F.M. conceived and designed research. L.M.D.N. and C. E. conducted experiments. C.C. and L.K. provided the biological material. L.M.D.N. and V.F. contributed new reagents and/or analytical tools. L.M.D.N., C.E. and V.F. performed the molecular work. L.M.D.N. and C.E. analyzed data. L.M.D.N., J.R. and L.K. contributed resources. L.M.D.N. and J.R. wrote the first draft of the manuscript. All authors revised the manuscript.

Data availability Sequences data that support the findings of this study have been deposited in the National Center for Biotechnology Information (NCBI) with the primary accession codes: PP946296, PP946297, PP946298, PP946299, PP964735, PP964737, PP964736, PP964734, PP964733.

Declarations

Competing interests The authors declare no competing interests.

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