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RESEARCH ARTICLE

Twenty-five years after: post-introduction association of *Mecinus janthinus* s.l. with invasive host toadflaxes *Linaria vulgaris* and *Linaria dalmatica* in North America

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Abstract

Linaria vulgaris, common or yellow toadflax, and *Linaria dalmatica*, Dalmatian toadflax (*Plantaginaceae*), are Eurasian perennial forbs invasive throughout temperate North America. These *Linaria* species have been the targets of classical biological control programmes in Canada and the USA since the 1960s. The first effective toadflax biological control agent, the stem-mining weevil *Mecinus janthinus* (*Coleoptera*: *Curculionidae*) was introduced from Europe in the 1990s. This weevil has become established on *L. dalmatica* and *L. vulgaris* in both countries, although it has shown greater success in controlling the former toadflax species. Genetic and ecological studies of native range *M. janthinus* populations revealed that weevils previously identified as a single species in fact include two cryptic species, now recognised as *M. janthinus*, associated with yellow toadflax, and the recently confirmed species *Mecinus janthiniformis*, associated with Dalmatian toadflax. The results of a comprehensive study characterising haplotype identities, distributions and frequencies within *M. janthinus* s.l. native range source populations were compared to those populations currently established in the USA and Canada. The presence of both *Mecinus* species in North America was confirmed, and revealed with a few exceptions a high and consistent level of host fidelity throughout the adopted and native ranges. Genetic analysis based on mitochondrial cytochrome oxidase subunit II gene (mt*COII*) defined the origin and records the subsequent North American establishment, by haplotype, of the European founder populations of *M. janthinus* (northern Switzerland and southern Germany) and *M. janthiniformis* (southern Macedonia), and provided population genetic indices for the studied populations. This analysis together with existing North American shipment receipt, release and rearing records elucidates probable redistribution routes and sources of both weevil species from initially released and established adopted range populations.

Introduction

Accurate taxonomic identification of biological control agents and their targets is critical for safe and effective classical biological control of non-native invasive plants (weeds). In recent years, advances in molecular techniques have facilitated a more detailed resolution of species boundaries, showing that many plant and arthropod species originally recognised on morphological

grounds as the same species, actually consist of species complexes that often include cryptic species (e.g. Madeira *et al.,* 2006; Toševski *et al.,* 2015; Vigalondo *et al.,* 2015). Such revelations can have major implications for the practice and success of weed biological control programmes, for example in the development of test plant lists for accurate, pre-release host range assessments of candidate biological control agents, or in correctly matching biological control agent species with their appropriate host plant species during agent releases (Gaskin *et al.,* 2011 and references therein). Here we report how an improved taxonomic and ecological understanding of one group of biological control agents has helped to explain highly variable outcomes of an established weed biological control programme. The goal of this study was to trace the origin of weevils released for classical biological control of yellow and Dalmatian toadflax that are now established in Canada and the USA to specific native range and host plant associated *Mecinus janthinus* s.l. source populations.

Yellow toadflax and Dalmatian toadflax, *Linaria vulgaris* Mill. and *Linaria dalmatica* (L.) Mill. (*Plantaginaceae*), are perennial Eurasian forbs that have become widespread invaders of natural areas and agricultural land in North America (Vujnovic & Wein, 1997; Pauchard *et al.,* 2003; Sing *et al.,* 2016). Both plants are listed as a noxious or regulated weed in British Columbia, Alberta, Saskatchewan, Manitoba, Idaho, Montana, Colorado, Nevada, New Mexico, Oregon, South Dakota, Washington and Wyoming (Center for Invasive Species Management, 2014; USDA-NRCS, 2018).

These two *Linaria* species have been the targets of classical biological control programmes in Canada and the USA since 1960s (De Clerck-Floate & Harris, 2002; McClay & De Clerck-Floate, 2002; De Clerck-Floate & McClay, 2013; De Clerck-Floate & Turner, 2013; Sing *et al.,* 2016). Host specificity data collected during 1989–99 by CABI, Delémont Switzerland (hereafter, CABI) on a stem-mining weevil then identified as *M. janthinus* Germar (*Coleoptera*: *Curculionidae*) (Jeanneret & Schroeder, 1992) supported releasing the weevil against both yellow toadflax and Dalmatian toadflax in North America. *M. janthinus* was approved for release in Canada against both *Linaria* species in 1991 by the Canadian Food Inspection Agency and subsequently approved for release in the USA in 1996 by American regulatory counterpart USDA APHIS PPQ. Although it initially appeared to have relatively little immediate impact on *L. vulgaris* infestations (McClay & De Clerck-Floate, 2002), the agent was credited with dramatic, landscape-level control of *L. dalmatica* in Canada (Van Hezewijk *et al.,* 2010), with similar impacts on *L. dalmatica* reported in the USA (Schat *et al.,* 2011; Jamieson *et al.,* 2012).

Assessments of the success of toadflax biological control became more complicated when Toševski *et al.* (2011) revealed that European field populations of the agent previously identified as a single species *M. janthinus*, in fact included two species, one now recognised as *M. janthinus* Germar, 1821 and the other a recently described cryptic sister species *Mecinus janthiniformis* Toševski & Caldara, 2011 (Toševski *et al.,* 2011). These can be distinguished by subtle morphological characters, but are only reliably identified through species-specific genetic differences in mitochondrial cytochrome oxidase subunit II sequences (mt*COII*) (Toševski *et al.,* 2011, 2013). In Europe, these species are ecologically partitioned, differing consistently (including in syntopy) in their host plant associations, and consequently, in their host-driven geographic distributions (Toševski *et al.,* 2011).

Most of the host specificity testing conducted to obtain approval for North American field release of *M. janthinus* (hereafter, *M. janthinus* s.l.) involved weevils collected on *L. dalmatica* (= *M. janthiniformis*) in Macedonia (Jeanneret & Schroeder, 1992). However, CABI's records show that the majority of weevils shipped to North America were collected from *L. vulgaris* in the Rhine Valley, Germany. This area is outside the known range of *M. janthiniformis*, so any shipments of weevils field collected from Rhine Valley yellow toadflax likely consisted solely of *M. janthinus* s.s. (Toševski *et al.,* 2011). At the time they were being introduced, all weevils shipped from Europe were described and accepted as one species equally effective against both invasive toadflax targets. Keeping imported, established or propagated *Mecinus* populations separate was therefore not prioritised. Potential management implications of the preceding factors have led us to question which of the two *Mecinus* species was released on, subsequently established on, and potentially redistributed from, specific North American sites currently or historically infested by either of these *Linaria* species. The sub-generic identity of agents released on the North American landscape is uncertain, and it is only with retrospective and current analysis that we can possibly determine where and which of the two *Mecinus* species was released and became established.

The introduction of exotic organisms for classical biological control programme facilitates, over time and across a range of novel environments, the opportunity to study ecological and population genetic responses of agent species. In a broader sense, introductions and episodic establishment precipitate ideal conditions to study the independent evolutionary pathways of agent populations, both those serving as the founder source in the native range, and their descendants in the introduced range. Though the use of classical biological control in the integrated management of invasive exotic

pests is now commonly practiced, population genetic responses of most agents in their introduced ranges is typically poorly known, albeit of fundamental importance to the practice and evaluation of biological control programmes.

Materials and methods

Tracing the origin of *M. janthinus* s.l. shipments to specific European collection sites

All available information related to shipments of weevils from Europe to North America made between 1991 and 1999 was evaluated to elucidate both pathways of introduction and sources of introduced populations of *M. janthinus* s.l. currently established in North America. Dry voucher specimens of individuals originally retained from weevil shipments were evaluated for confirmation of species status using molecular tools. The primary archival sources of information on the origin of weevils introduced from Europe to North America were CABI shipment forms and documentation from government agencies that received live insects for toadflax biological control release programmes in Canada and the USA. Documentation included information on the origin of the weevils, such as collection and/or shipment date(s), number and life stage of weevils in each shipment, location of collection site(s) in Europe, species of plant(s) weevils were collected from, and receiving destination/facility in Canada or the USA. In addition, all available data tracking the North American dissemination of weevils in each shipment, from the receiving containment/propagation facility to their final field release destinations, was included.

Acronyms

The collections housing the material studied in this paper are abbreviated as follows:

- CABIC – CABI collection, Delémont, Switzerland
- AGRL – Agriculture and Agri-Food Canada, Lethbridge
- ITCB – Ivo Toševski collection, Belgrade, Serbia

Insect sampling

Previous studies of haplotype diversity associated with the mitochondrial *COII* gene in European *M. janthinus* s.l. populations revealed a total of 20 *M. janthinus* haplotypes associated with *L. vulgaris* (v1–v20), 29 *M. janthiniformis* haplotypes associated with *Linaria genistifolia*/*L. dalmatica* (g1–g13, d1–d12, gd1–gd4) and 3 haplotypes designated as the 'speciosa' group genotype associated with *L. vulgaris* (s1–s3) (Toševski *et al.,* 2011, 2013). Weevils used in the present study were similarly field collected from *L. vulgaris*, *L. genistifolia* and *L. dalmatica* at 64 native range locations (Fig. 1). To take into consideration the metapopulation organisation of European *M. janthinus* s.l. populations, field collected specimens for the present study were obtained through more intensive sampling of the entire European range of *M. janthinus* s.l., and not limited to source populations of the 1991–99 weevil shipments received by North American toadflax biological control programmes (Tables S1 and S2, Supporting information). A total of 155 *M. janthinus* from 27 locations (Table S3) were sequenced to determine native range haplotype diversity of the yellow toadflax stem miner. In addition, 246 *M. janthiniformis* from 37 southeastern European locations (Table S4) were analysed: 81 specimens associated with *L. genistifolia* (20 locations) and 161 specimens associated with *L. dalmatica* ssp. *macedonica* from southern Macedonia (10 locations) and northern Greece (5 locations), and 4 specimens associated with *L. dalmatica* ssp. *dalmatica* from 2 locations in Montenegro.

Molecular diagnostics were performed on 632 individuals sampled from 66 locations where *M. janthinus* s.l. populations were established in North America. A total of 224 specimens were collected in association with *L. vulgaris* and 403 specimens in association with *L. dalmatica*, from 14 and 52 locations, respectively (Fig. 2). The presence of *L. genistifolia* in North America has not yet been molecularly confirmed (Sing *et al.,* 2016), so specimens were collected in Canada and the USA from two toadflax species, *L. vulgaris* and *L. dalmatica*, and from their naturally occurring hybrids (Ward *et al.,* 2009). In many cases, specimens for this analysis were obtained through host plant stem dissections, where all development from oviposition through adult eclosion was completed. Specimens for analysis were also obtained as emerged adult weevils hand collected externally on toadflax plants. This method was used for collecting weevils on North American field sites where *M. janthinus* s.l. had previously been released, particularly where releases involved isolated, single toadflax species infestations. Emerged adult samples were taken as close as possible to documented original release locations. Specimens from populations distributed in Europe and those now established in North America were field collected between 2006 and 2016. All specimens were stored individually in 96% ethyl alcohol at 4°C. For European collections, attention was focused on sampling populations of *M. janthinus* and *M. janthiniformis* that served as likely sources for North American introductions/releases made between 1 July 1991 and 25 May 1999. Voucher specimens analysed in this study are deposited in either the ITCB or AGRL collections.

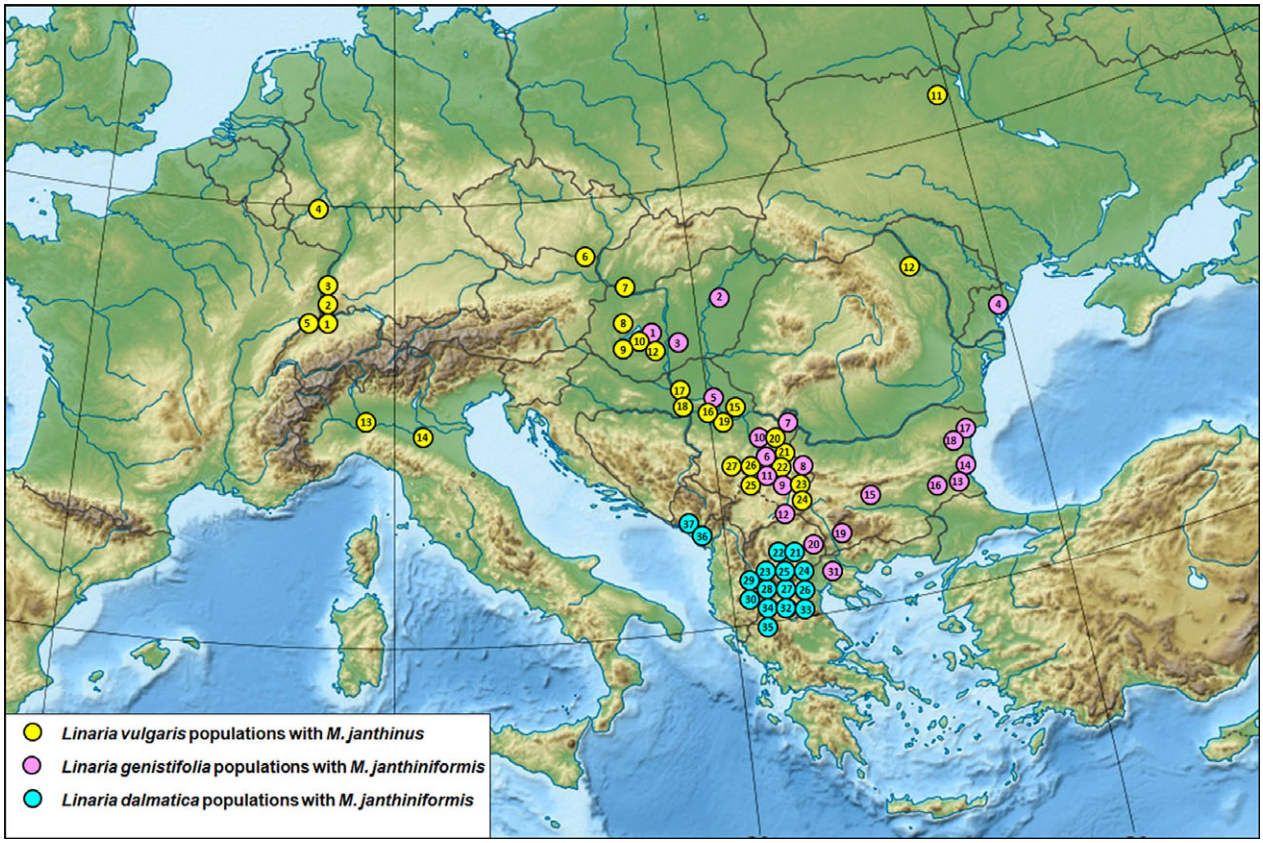

Figure 1 Location of sampling sites in Europe for *Mecinus janthinus* s.l. specimens collected from *Linaria vulgaris* (yellow circles), *Linaria genistifolia* (pink circles) and *Linaria dalmatica* (blue circles). Numbers inside the circles indicate the reference locations listed in Tables S3 and S4.

DNA extraction

Field collected weevils were individually punctured at the second thoracic sternite and total DNA was extracted using the Qiagen Dneasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The same procedure was used to extract DNA from 27 dry voucher specimens of *M. janthinus* s.l. Specimens analysed were individuals drawn from shipments sent to Canada during 1991–96, from weevil populations used in CABI host specificity tests, or from weevils, field collected from early established populations in Canada, and deposited at CABIC or AGRL. Following DNA extraction, voucher specimens were labelled and returned to holder institutions.

Polymerase chain reaction amplification and sequencing

Field specimen cytochrome oxidase subunit II gene (*COII*) was amplified using the primers TL2-J-3038 (5′-TAATATGGCAGATTAGTGCATTGGA-3′) (Emerson *et al.*, 2000) and TK-N 3782 (5′-GAGACCATTACTTGCTT TCAGTCATCT-3′) (Harrison Laboratory, Cornell University, Ithaca, NY, USA). Amplification reactions were performed in a 20 μL final reaction volume containing Kapabiosystems High Yield Reaction Buffer-A with 1.5 mM $MgCl_2$ (1×), an additional 3.5 mM of $MgCl_2$, 0.8 mM of each dNTP, 0.75 μM of each primer, 0.75 U of KAPA*Taq* DNA polymerase (Kapa Biosystems, Inc., Woburn, MA, USA) and 1 μL of DNA extract. Polymerase chain reactions (PCRs) were carried out in a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany), applying the following thermal steps: 95°C for 5 min (initial denaturation), and 40 cycles at 95°C for 1 min, 1 min at 45°C (annealing), 72°C for 2 min, and a final extension at 72°C for 10 min. PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and sequenced by BMR Service (Padova, Italy) and Macrogen (Seoul, Korea). For most specimens, sequences of the complete *COII* gene were obtained with the forward primer only; for more recalcitrant specimens, sequencing was performed with both primers of full-length PCR products. Sequences are available from GenBank under

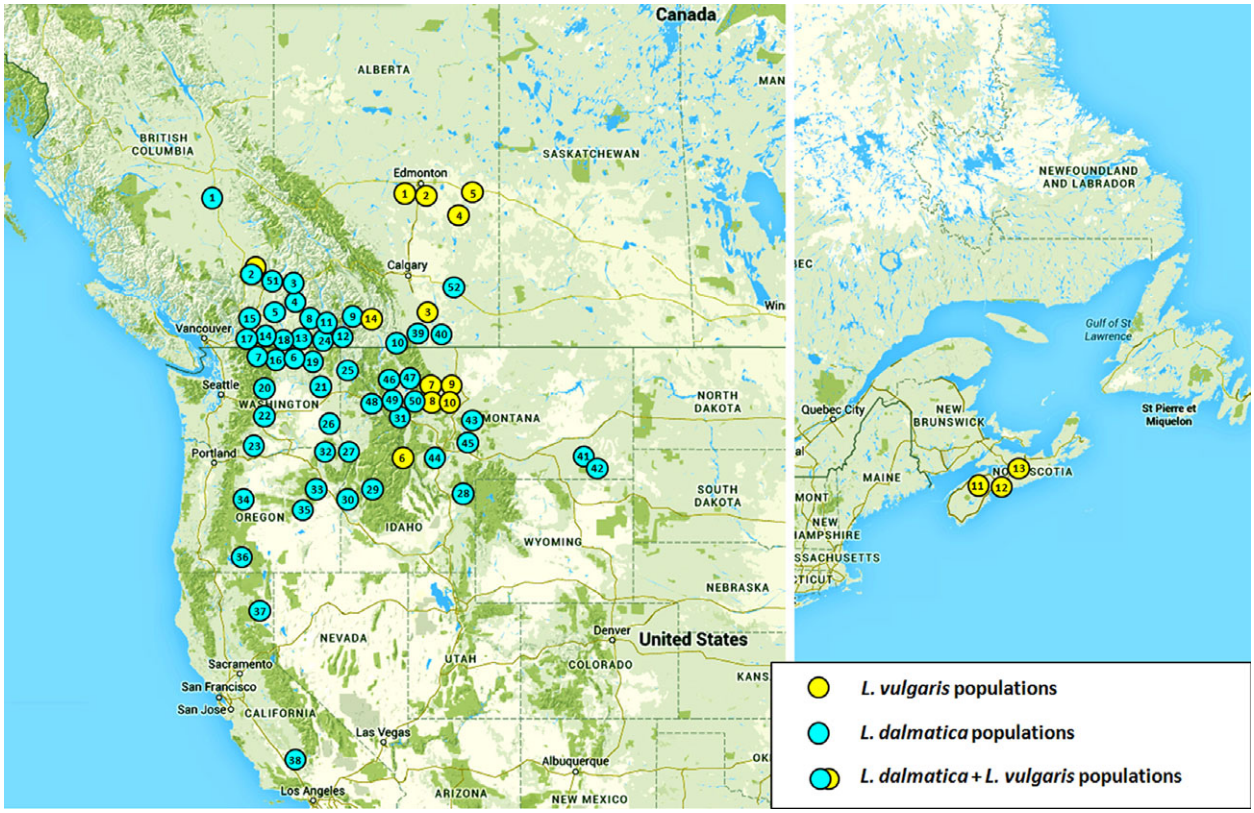

Figure 2 Location of sampling sites in North America for *Mecinus janthinus* s.l. specimens collected from *Linaria vulgaris* (yellow circles), and *Linaria dalmatica* (blue circles). Numbers inside the circles indicate the reference locations listed in Tables S5, S6 and S7.

accession numbers JN037471–JN037662 (Toševski *et al.*, 2011), JX631141–JX631145, JX631146–JX631152, JX631153–JX631155 (Toševski *et al.*, 2013) and MF068710–MF068741 (this study) for the *COII* gene.

For dry voucher specimens, a set of three primer pairs was used according to Toševski *et al.* (2011) for specific amplification of short mitochondrial *COII* fragments (less than 100 bp in length), to account for the frequent highly degraded and fragmented state of mitochondrial DNA (mtDNA) in dry insect material. Primers used for the amplification of these short mtDNA fragments were positioned within the *COII* gene sequence so they would amplify regions of the gene previously determined to be informative and specific for host–plant association of the *M. janthinus* s.l. specimens, which is at positions 171, 174, 252 and 489 of the *COII* gene. For amplification of short mitochondrial fragments, the PCR mixture was the same as described for the full-length *COII* amplicon, and all thermal steps were identical except that the extension time was reduced to 30 s and final extension time to 3 min. PCR products of short fragments were cleaned as described above and sequenced with a reverse and forward tail adaptor (Toševski *et al.*, 2011).

Haplotype network construction

Maternally inherited mitochondrial DNA was determined to be the most suitable attribute to use to compare haplotype diversity/similarity of European with established North American populations of the introduced weevils, according to their association with specific invasive toadflax populations. Two *Mecinus* species, the weed biological control agent intended for release (*M. janthinus*) and a closely related cryptic sister species (*M. janthiniformis*), were hypothesised to be introduced to North America within a relatively short period of time (Toševski *et al.*, 2011). Relationships between the mitochondrial content (*COII* gene) of ancestral populations originating from Europe and offspring populations established in North America are better visualised in reticulate graphs or networks (Posada & Crandall, 2001), which would additionally provide quantifications for successfully established genotypes. Thus, TCS version 1.21 (Clement *et al.*, 2000) was employed to infer haplotype networks among European and North American haplotypes using statistical parsimony (Templeton *et al.*, 1992) with a confidence limit of 95%.

Analysis of molecular variance and population indices based on mt*COII* gene diversity of *M. janthinus* s.l. populations in Europe and North America

Analysis of molecular variance (AMOVA) of mt*COII* was performed on *M. janthinus* s.l. populations in Arlequin 3.5 (Laurent Excoffier, Institute of Ecology and Evolution, University of Berne) (Excoffier & Lischer, 2010). Association of genetic variation with different hierarchical levels was tested on European populations of *M. janthinus* (ex. *L. vulgaris*) and *M. janthiniformis* (ex. *L. genistifolia*/*L. dalmatica*) grouped by country or geographical region within country, and on North American weevil populations grouped according to state or provincial distribution. Standard population indices, that is haplotype and nucleotide diversity, for *M. janthinus* and *M. janthiniformis* founder and introduced populations were used to assess and compare levels of polymorphism. Arlequin was also employed to determine levels of differentiation between grouped populations of both *Mecinus* species, using population pairwise F_{ST} analysis. The resulting matrix of F_{ST} values was used to visualise genetic distances between paired populations. In addition, neutrality test statistics (Tajima's *D*, Fu's *Fs*) were used to detect any departure from neutrality resulting from selection or demographic processes.

Results

Origin of European *M. janthinus* s.l. in specific shipments to North America

According to accompanying shipping records deposited in CABI's archives, CABI sent 20 shipments of 1800 total adult weevils to containment facilities in Canada (Agriculture Canada Research Station, Regina, SK; Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, Lethbridge, AB) and the USA (Montana State University, Bozeman, MT; USDA APHIS CPHST Mission Biological Control Laboratory, Edinburg, TX) between 1991 and 1999 (Table S1). Seventeen of the 20 shipments contained weevils collected only from yellow toadflax in the Rhine Valley, Germany. Three shipments contained weevils collected at other locations, either from mixed stands of Dalmatian toadflax and yellow toadflax, or from a Macedonian subspecies of Dalmatian toadflax (i.e. *L. dalmatica* ssp. *macedonica*) (Table S1). A single shipment to Montana State University in 1993 of 22 adults originating from Romania, Bulgaria and Greece was not released or propagated for release in containment, but used only in electrophoretic studies; adults from this shipment were therefore not included in the present study. A retrospective analysis of archival shipping documents indicates that all *Mecinus* spp. established in North America today originated from 1555 weevils collected on yellow toadflax in the Rhine Valley, Germany; 30 weevils collected from a mixed Dalmatian–yellow toadflax stand in Hungary; and 193 weevils collected from *L. dalmatica* ssp. *macedonica* in southern Macedonia.

Analysis of dry voucher specimens

Twenty-seven dry voucher specimens from CABI or AGRL collections (Table S2) were sequenced. All five voucher specimens taken from the first CABI shipment to Canada (1991) were confirmed through sequencing to be *M. janthinus*, although each had been labelled as collected from *L. vulgaris* in the Rhine Valley, and from *L. vulgaris* and *L. dalmatica* in Hungary. Two pools of weevils were drawn in 1996 as voucher specimens from CABI's resident toadflax host specificity testing populations. Eight weevils labelled '*L. vulgaris* test population, Rhine Valley' were all confirmed to be *M. janthinus*. Sequencing of a second pool of four specimens labelled as '*L. vulgaris*/*L. dalmatica* test population, Macedonia' identified two specimens as *M. janthinus* and two as *M. janthiniformis* (Table S2).

Tests were also performed on 10 voucher specimens deposited in the Agriculture and Agri-Food Canada (AGRL) collection (Table S2, last two groups). All individuals sequenced were collected on Dalmatian toadflax and were drawn from *M. janthinus* s.l. populations at two sites where this agent first became established in Canada. Five specimens collected in 1994 from a garden insectary used for propagating toadflax biological control agents in Kamloops BC were confirmed to be *M. janthiniformis* (Table S2). Five specimens collected from Dalmatian toadflax in 1992 on the 'Scandia' release site near Brooks, AB, were identified as *M. janthinus* (Table S2). Molecularly based taxonomic verification of the voucher specimens originating from laboratory, propagation garden and field release populations unequivocally confirmed the presence of two *Mecinus* species within the tested material, *M. janthinus* and *M. janthiniformis*. DNA sequencing of the Kamloops insectary site dry voucher samples collected in 1994 confirms that despite limited introduction opportunities, restricted to only a 1991 mixed population shipment of 30 weevils, and a subsequent shipment of 193 Macedonian weevils in 1992 (Table S1), *M. janthiniformis* was established in North America 3 years after the first possible release.

Haplotype diversity inferred from the *mtCOII* gene in European populations of *M. janthinus* and *M. janthiniformis*

Analysis of the material sampled from a broader area of the *M. janthinus* s.l. native range revealed the existence of

Table 1 European and North American frequencies and distributions of *Mecinus janthinus* haplotypes established on *Linaria vulgaris* in North America

Haplotype Name	Frequency in NA[a] (%)	Frequency in Europe[a] (%)	Distribution in Europe	Distribution in NA	Host Plant[b]
v1	56 (27.9)	27 (30.0)	CH, D	AB, BC, ID, MT, NS	*L. vulgaris*^{EU}; *L. vulgaris*^{NA}; *L. dalmatica*^{NA}
v4	116 (57.7)	14 (16.1)	CH, D	AB, ID, MT, NS	*L. vulgaris*^{EU}; *L. vulgaris*^{NA}
v23	2 (1.0)	2 (2.3)	D	AB	*L. vulgaris*^{EU}; *L. dalmatica*^{NA}
v1NA	22 (10.9)	–	–	AB, NS	*L. vulgaris*^{NA}
v2NA	4 (2.0)	–	–	AB, MT	*L. vulgaris*^{NA}
v3NA	1 (0.5)	–	–	AB	*L. vulgaris*^{NA}

AB, Alberta; BC, British Columbia; CH, Switzerland; D, Germany; ID, Idaho; MT, Montana; NA, North America; NS, Nova Scotia.
[a]Frequencies calculated according to the total number of sequenced specimens from all studied populations in North America ($n = 201$) and founder populations in Europe ($n = 87$).
[b]Host plant in Europe^{EU} and North America^{NA}.

five previously unreported haplotypes within *M. janthinus* s.s.: two haplotypes (v21 and v22) from Hungary (Balaton Lake), and three new haplotypes (v23–v25) from southern Germany (Eschbach) (Table S3). Within *M. janthiniformis*, eight additional haplotypes were recorded in weevils associated with *L. dalmatica* (d13–d20), and one more associated with *L. genistifolia* (Table S4). The *M. janthiniformis* haplotype g11 previously recorded from Primorsko (Bulgaria) in association with *L. genistifolia* (Toševski *et al.*, 2013) was also recorded in the present study from Mariovo (southern Macedonia), in association with *L. dalmatica* ssp. *macedonica* (Table S4). Therefore, to stabilise the nomenclature of the *M. janthiniformis* haplotypes, haplotype g11 was renamed gd5. This expanded molecular analysis of native range *M. janthinus* s.l. increases the known haplotype diversity, from 20 to 22 haplotypes recorded within *M. janthinus* populations associated with *L. vulgaris* (Table S3), and from 29 to 38 haplotypes within *M. janthiniformis* populations associated with *L. genistifolia* and *L. dalmatica* (Table S4). No new haplotypes were recorded within the 'speciosa' group genotype (Toševski *et al.*, 2011).

Haplotype inference based on *mtCOII* gene in North American populations of *M. janthinus* s.l. associated with yellow toadflax and Dalmatian toadflax

Sequencing of 201 weevils collected from North American *L. vulgaris* identified the presence of five haplotypes, two of those common to European and North American sequenced specimens (Tables 1 and S5). These were *M. janthinus* European haplotypes v1 and v4, exclusively distributed in southern Germany and northern Switzerland (Rhine Valley) (Table S3). In addition, two specimens recorded on *L. dalmatica* from Scandia AB (Table S7), but from no other sampled locations in North America, were consistent with European *M. janthinus* haplotype v23, distributed only in southern Germany (Table S3). Haplotypes v4 and v1 were the most abundant among the sequenced North American weevils, and detected at a frequency of 57.7% and 27.9%, respectively (Table 1). Haplotype v23 was detected at a similarly low frequency both in the native and adopted ranges.

The remaining three *M. janthinus* haplotypes recorded in North American specimens were designated as v1NA, v2NA and v3NA because they were detected only within North American populations of *M. janthinus*. Haplotype v1NA was detected at the third highest frequency (10.9%) in all North American *M. janthinus* specimens sequenced, recorded in 22 specimens originating from three locations in Alberta (Edmonton, Rosalind and Kinsella) and two locations in Nova Scotia (St. Croix and Valley, Colchester Co.) (Table 1). Only four specimens with the v2NA haplotype were recorded, two each from Kinsella AB and Ovando MT. The v3NA haplotype was recorded once, from a specimen collected in 2016 on *L. vulgaris* in a remnant outdoor mixed *L. vulgaris*/*L. dalmatica* propagation plot at AAFC, Lethbridge AB. Established populations of *M. janthinus* in North America averaged 2.2 ± 0.8 (range: 1–4, $n = 13$) haplotypes per site.

Sequencing of the 431 *M. janthiniformis* specimens from North America revealed a total of 12 haplotypes, with nine common between European and North American sequenced specimens (Tables 2 and S6). Five haplotypes recorded from North American specimens corresponded to *M. janthiniformis* European haplotypes d1, d3, d5, d9 and d14, exclusively associated with *L. dalmatica* ssp. *macedonica* in southern Macedonia and northern Greece. Four haplotypes recorded from North American specimens corresponded to European haplotypes associated with both *L. genistifolia* and *L. dalmatica* ssp. *macedonica*: gd1, gd3, gd4 and gd5. Haplotypes gd4 and d9 were the most abundant among the sequenced North American weevils, and detected at a frequency of 28.5% and 18.3%, respectively (Table 2). The frequency with which haplotypes d3, d5, d9, d14 and gd5 were detected was substantially higher in North American than European specimens (Table 2). The most abundant and widespread *M. janthiniformis* haplotype recovered so far in North America, gd4,

Table 2 European and North American frequencies and distributions of *Mecinus janthiniformis* haplotypes established on *Linaria dalmatica* in North America

Haplotype Name	Frequency in NA[a] (%)	Frequency in Europe[a] (%)	Distribution in Europe	Distribution in NA	Host Plant[b]
gd1	1 (0.2)	15 (9.3)	MK, GR, BG, SRB	MT	*L. dalmatica* ssp. *macedonica*^{EU}; *L. genistifolia*^{EU}; *L. dalmatica*^{NA}
gd3	1 (0.2)	7 (4.3)	MK, GR, SRB	MT	*L. dalmatica* ssp. *macedonica*^{EU}; *L. genistifolia*^{EU}; *L. dalmatica*^{NA}
gd4	123 (28.5)	45 (27.9)	MK, GR,	BC, WA, OR, ID, MT, CA, NS	*L. dalmatica* ssp. *macedonica*^{EU}; *L. genistifolia*^{EU}; *L. dalmatica*^{NA}; *L. vulgaris*^{NA}
gd5	26 (6.0)	1 (0.6)	MK, BG	BC, WA, OR, ID	*L. dalmatica* ssp. *macedonica*^{EU}; *L. genistifolia*^{EU}; *L. dalmatica*^{NA}
d1	15 (3.5)	17 (10.6)	GR	BC, MT	*L. dalmatica* ssp. *macedonica*^{EU}; *L. dalmatica*^{NA}; *L. vulgaris*^{NA}
d3	62 (14.4)	4 (2.5)	MK	BC, OR, ID, WA, CA	*L. dalmatica* ssp. *macedonica*^{EU}; *L. dalmatica*^{NA}
d5	43 (10.0)	6 (3.7)	MK, GR	BC, WA, OR, ID, CA	*L. dalmatica* ssp. *macedonica*^{EU}; *L. dalmatica*^{NA}; *L. vulgaris*^{NA}
d9	79 (18.3)	2 (1.2)	GR	BC, WA, OR, ID, MT, CA, NS	*L. dalmatica* ssp. *macedonica*^{EU}; *L. dalmatica*^{NA}; *L. vulgaris*^{NA}
d14	44 (10.2)	1 (0.6)	MK	BC, WA, OR, ID,	*L. dalmatica* ssp. *macedonica*^{EU}; *L. dalmatica*^{NA}; *L. vulgaris*^{NA}
d1NA	29 (6.7)	–	–	BC, WA, OR, ID, MT, CA	*L. dalmatica*^{NA}; *L. vulgaris*^{NA}
d2NA	7 (1.6)	–	–	OR, ID, MT	*L. dalmatica*^{NA}
d3NA	1 (0.2)	–	–	ID	*L. dalmatica*^{NA}

BC, British Columbia; BG, Bulgaria; CA, California; GR, Greece; ID, Idaho; MK, Macedonia; MT, Montana; NA, North America; NS, Nova Scotia; OR, Oregon; SRB, Serbia; WA, Washington.

[a]Frequencies calculated according to the total number of sequenced specimens from all studied populations in North America ($n = 431$) and founder populations in Europe ($n = 161$).

[b]Host plant in Europe^{EU} and North America^{NA}.

registered in 28.5% of sequenced specimens, was also the most frequently detected European haplotype (27.9%) among the nine confirmed haplotypes common to Europe and North America. Haplotype d9 was registered as rare in the sequenced specimens of *M. janthiniformis* from Europe (1.2%), but was very common within sampled North American populations of this species, recorded in 79 sequenced specimens (18.3%) and in 34 out of 51 studied populations. The occurrence of three *M. janthiniformis* haplotypes in North America, gd1, gd3 and gd5 (of the nine common to both the native and adopted ranges), was inversely proportional to the frequency with which they were detected in the native range. Haplotype gd1 was abundant and widely distributed in its southeastern European native range, recorded from 15 locations including Serbia (3), Bulgaria (6), Macedonia (2) and Greece (4), and is associated with both *L. genistifolia* (Serbia and Bulgaria) and *L. dalmatica* ssp. *macedonica* (Macedonia and Greece), but registered with a frequency of only 0.2% in North American specimens (Table 2).

Haplotypes d1NA, d2NA and d3NA were not consistent with any of the 38 haplotypes identified within European *M. janthiniformis* populations associated with *L. genistifolia* and/or *L. dalmatica*. One of the three *M. janthiniformis* haplotypes recorded only in North America, haplotype d1NA, was relatively abundant (6.7%) and widely distributed, found at 13 locations (BC, WA, OR, ID, MT and CA) (Table 2). Haplotype d2NA was recorded in only 7 of 431 sequenced specimens (1.6%), but present at five locations: Arrow Junction ID; Missoula, Beaverhead, Bison Range 2, all in Montana; and Susanville CA (Table S6). Haplotype d3NA was recorded in a single specimen from a confirmed hybrid toadflax infestation in Palisades ID (Table S6) (Boswell *et al.,* 2016). Regarding haplotype richness of established populations of *M. janthiniformis* in North America, most populations contained diverse haplotypes, averaging 4.1 ± 1.2 (range: 1–6, $n = 51$) haplotypes per site.

In a few cases, *M. janthinus* s.l. haplotypes of North American specimens were recorded in association with atypical hosts, that is *M. janthinus* with *L. dalmatica* and *M. janthiniformis* with *L. vulgaris*. This discrepancy was never recorded for the natural native hosts in European populations of the respective weevil species (Toševski *et al.,* 2011, 2013). One specimen carrying haplotype v1 corresponding to an *M. janthinus* mitochondrial genotype was recorded within a predominantly *M. janthiniformis* population propagated since 1992 on Dalmatian toadflax at Kamloops BC (Table S7). *M. janthiniformis* was recorded at six locations in association with *L. vulgaris*, which is not the natural host for European populations of this species (Table S7). Eight out of 33 sequenced specimens collected

on yellow toadflax in Salmon ID were confirmed to be *M. janthiniformis*, and all corresponded to the unique North American haplotype d1NA (Table S7). The d1NA *M. janthiniformis* haplotype was also recorded in 3 out of 52 specimens collected from *L. vulgaris* in Ovando, MT. In addition, 3 out of 4 specimens collected from yellow toadflax at one site in Edmonton AB were *M. janthiniformis* haplotype gd4. Finally, 5 out of 13 sequenced specimens from a population associated with yellow toadflax in St. Croix NS were identified as the gd4 and d9 haplotypes of *M. janthiniformis*. *M. janthiniformis* haplotypes were also identified from two different sites infested by two different types of hybrid toadflax. Nine sequenced specimens collected in Jaffray BC from hybrids of *L. dalmatica* and *L. vulgaris* (J. Gaskin & I. Toševski, unpublished data) were identified as four haplotypes of *M. janthiniformis*: d1, d5, d9 and d14. The 35 sequenced specimens collected from hybrids of *L. dalmatica* and *L. vulgaris* infesting the Palisades ID site were all identified as *M. janthiniformis* haplotypes: d3, d5, d9, gd4, gd5 and d3NA (Table S6).

Genealogy of mt*COII* genes in European and North American *M. janthinus* s.l. populations

Haplotype networking using statistical parsimony based on the full length of *COII* genes allowed for simultaneous visualisation of all haplotypes recorded in European and North American populations for both *Mecinus* species, as well as for the genealogy and origin of each (Fig. 3). According to haplotype distributions across the area surveyed in Europe, 13 haplotypes, that is v1–v7, v17–v19 and v23–v25, all recorded from *L. vulgaris* in the Rhine Valley area, could be considered the gene pool for the founder population of *M. janthinus* in North America during the 1991–99 release period. Although a total of 1585 *M. janthinus* adults collected from Rhine Valley *L. vulgaris* were introduced through repeated releases over a period of 9 years, only 3 of the 13 haplotypes found within this European collection region became established in North America (Tables 1 and S5). None of the 'speciosa' genotypes were detected from 201 sequenced specimens of North American field collected *M. janthinus*.

The European origins of the founder population of *M. janthiniformis* now established in North America are locations in southern Macedonia inhabited by *L. dalmatica* ssp. *macedonica*. This agrees with supporting documentation related to weevils collected from four locations between the towns of Prilep and Bitola in southern Macedonia, close to the border with Greece, and shipped in 1992 to Regina SK (Table S1). The present study spanned a wider area of the host plant's distribution in Macedonia and northern Greece than where weevils were collected for the 1992 shipment, to account for the possibility that the material sampled for analysis represents an *M. janthiniformis* metapopulation. Twenty-five *M. janthiniformis* ex. *L. dalmatica* ssp. *macedonica* haplotypes were identified: gd1–gd5 and d1–d20. The result of extensive sampling (Fig. 4), this finding could represent an integral part of the founder population of *M. janthiniformis* in North America, because all haplotypes now established in Canada and the USA originated from one collection made in the same area, in 1992. Nine *M. janthiniformis* haplotypes established in North America represent 36% of the all haplotypes recorded from the locations in Macedonia where weevils were collected in 1992. To complete the data set of haplotype diversity we therefore expanded sampling of *M. janthiniformis* adults to the entire distribution range of *L. dalmatica* ssp. *macedonica* in southern Macedonia and northern Greece. According to the analysis based on sequenced adults from the founder population, that is four locations in southern Macedonia, as well as from the expanded range of *M. janthiniformis* in this region (Fig. 4), a total of 25 haplotypes of *M. janthiniformis* were found to be associated with *L. dalmatica* ssp. *macedonica*. Five of the registered haplotypes (gd1–gd5) were also associated with *L. genistifolia* from which gd1 and gd2 were recorded as common and as the most widely distributed *M. janthiniformis* haplotypes in southeastern Europe. In contrast, haplotype gd4 was frequent on nearly all sites with *L. dalmatica* ssp. *macedonica* but within a limited geographic range, and present only in Macedonia and Greece. Haplotype gd3 was less frequent and its distribution range was limited to southern Serbia, Macedonia and northern Greece (Table S4). Nevertheless, four genotypes from this group were recorded in North America, the most common haplotype gd4, and the rarest haplotype gd5, at frequencies of 28.5% and 6.0%, respectively. Haplotype gd1 with a frequency of 9.3% in the founder populations was recorded only in a single sequenced specimen representing a frequency of 0.2%, from the Crow 2 (MT) site.

No reticulation was observed in the haplotype network of *M. janthinus* haplotypes sampled in Europe, while a single reticulation event was recorded in the network of European haplotypes for *M. janthiniformis* (Fig. 3A). In contrast, two more reticulations were observed when haplotypes recorded only in North America of both *Mecinus* species were included in the combined network analysis (Fig. 3B).

Analysis of molecular variance and population indices based on *mtCOII* gene diversity of *M. janthinus* s.l. populations in Europe and North America

Based on the AMOVA results with populations grouped according to geographic distribution, only 15.1% and 3.7% of genetic variation are attributed to grouping

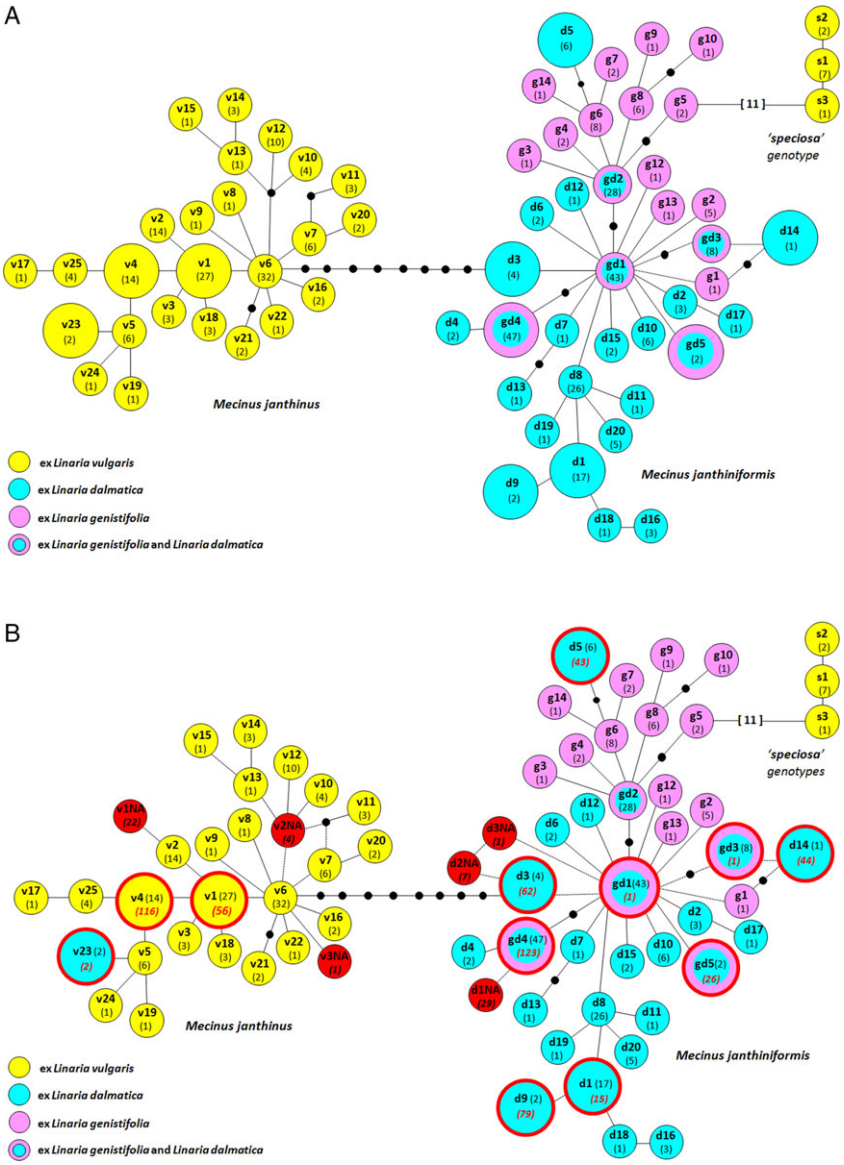

Figure 3 Statistical parsimony network based on cytochrome oxidase subunit II (*COII*) sequences of *Mecinus janthinus* s.l.: (A) haplotypes recorded in European populations of *M. janthinus* and *M. janthiniformis*; (B) haplotypes recorded in European and North American populations of *M. janthinus* and *M. janthiniformis*. Colours and letters inside the circles correspond to weevil mitochondrial lineages associated with different host plants: v – *Linaria vulgaris*, yellow; g – *Linaria genistifolia*, pink; d – *Linaria dalmatica*, blue. Common haplotypes associated with *L. genistifolia* and *L. dalmatica* are labelled gd within pink and blue circles. Circle sizes are not proportional to haplotype frequency. Large circles outlined in red represent haplotypes recorded both in Europe and North America. Solid red circles represent haplotypes recorded only in North America. Small black circles represent missing or unsampled haplotypes. Haplotype frequency is listed inside brackets, in black, regular font for European, and in red italics for North America haplotypes. Number in square brackets represents the number of mutations connecting '*speciosa*' genotypes associated with *L. vulgaris*.

between EU and NA populations of *M. janthinus* and *M. janthiniformis*, respectively (Table S8). Most of the genetic variation, 61.6% for *M. janthinus* and 86.3% for *M. janthiniformis*, is explained by variation within sampled populations.

Pairwise comparisons between populations of *M. janthinus* revealed a low but significant level of differentiation between the Switzerland/Germany founder populations and sampled conspecific North American populations. Pairwise F_{ST} comparison of the European founder with sampled North American *M. janthinus* populations revealed values ranging from 0.036 ($P < 0.001$) for Nova Scotia to 0.039 ($P < 0.01$) for Alberta, and 0.09 ($P < 0.001$) for Idaho to 0.16 ($P < 0.001$) for

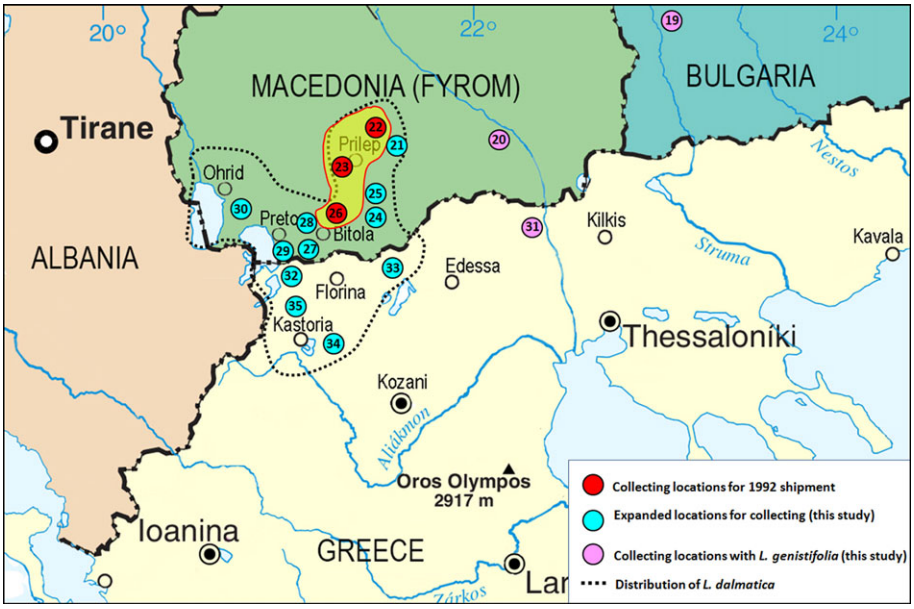

Figure 4 The 1992 collection sites for *Mecinus janthiniformis* in southern Macedonia (red circles), representing the probable source of founder populations for haplotypes detected in North American populations. Sampling area for the present study was expanded to include additional locations within the distribution of *Linaria dalmatica* ssp. *macedonica* (blue circles) and *Linaria genistifolia* (pink circles).

Montana (Fig. 5 and Table S9). Additionally, the Switzerland/Germany founder populations exhibited high and significant differentiation from other *M. janthinus* populations sampled in Europe, with F_{ST} values ranging from 0.38 to 0.51 ($P < 0.001$ for all values).

Pairwise comparisons between populations of *M. janthiniformis* similarly revealed a low but significant level of genetic differentiation between the southern Macedonia founder population and sampled North American populations (Fig. 6 and Table S10). Levels of differentiation between the European founder and sampled North American populations of *M. janthiniformis* in Canada ranged from 0.034 ($P < 0.001$) to 0.065 ($P = 0.063$), and in the USA from 0.032 ($P < 0.001$) to 0.129 ($P < 0.001$). Results also confirmed differentiation of the European founder population of *M. janthiniformis* from sampled conspecific European populations, with F_{ST} values for pairwise comparisons ranging from 0.07 ($P = 0.054$) to 0.47 ($P < 0.001$).

Population genetic indices for North American *M. janthinus* populations, indicated by values for nucleotide (0.001485) and haplotype (0.5797) diversity, were lower compared to the European founder population (0.002272 and 0.8215, respectively). Mean number of pairwise differences were similarly lower for the North American populations (1.007065) than for the European founder population (1.540506) (Table S11). In contrast, North American populations of *M. janthiniformis* presented higher levels of polymorphism than the European founder population, with values for haplotype (0.8315) and nucleotide (0.005003) diversity in North American populations higher than those for the European founder population (0.7386 and 0.003274, respectively).

The results of neutrality tests indicated by Fu's *Fs* statistic were significant and negative for European populations of both *M. janthinus* and *M. janthiniformis* (Table S11). However, North American populations of both species did not exhibit significant levels of departure from neutrality. Additionally, when Tajima's *D* parameter was tested based on the frequency of the segregating sites, no significant results were obtained for either *Mecinus* species.

Discussion

We presented in this study an example of how an in-depth assessment of an established biological control programme can be conducted retrospectively, using historic documentation and modern molecular techniques, to reveal the influence of pathways of introduction on the current population genetics of two closely related and widely distributed weed biological control agents. DNA sequencing of *M. janthinus* s.l. populations provided a *post hoc* opportunity to study population genetic indices of introduced weevils, for comparison to those from native range populations. The outcomes, in our case how the agent *M. janthinus* s.l. was introduced to North America and the haplotypes that successfully established from

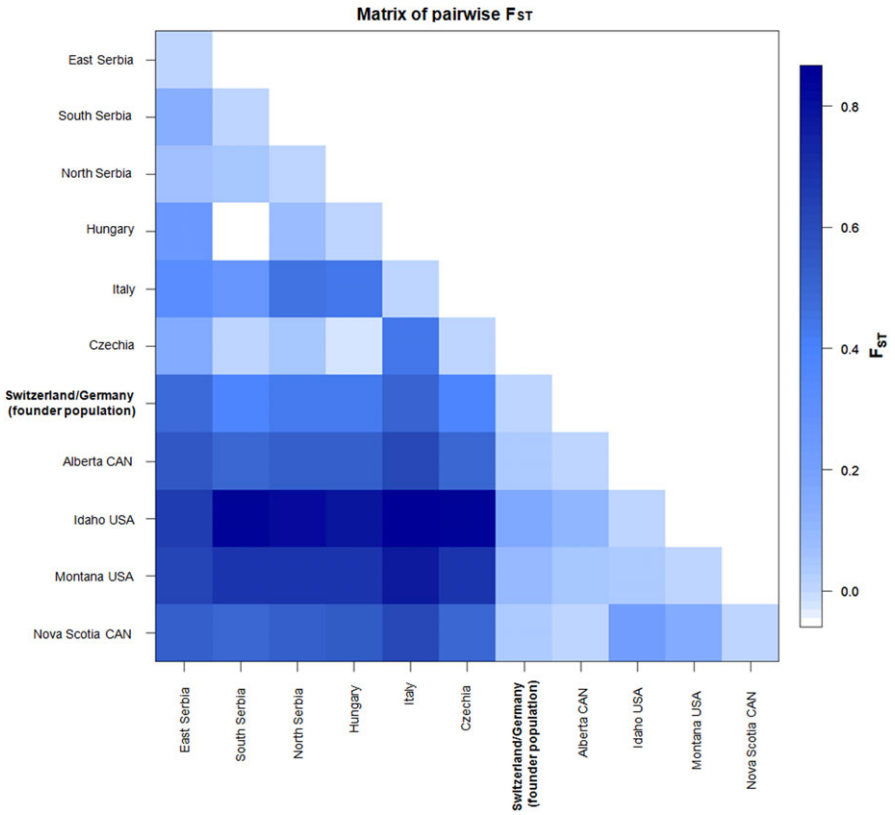

Figure 5 Matrix of pairwise mt*COI*-based F_{ST} for European and North American *Mecinus janthinus* populations, sorted by geographical distribution (regions, provinces or states). Populations are designated on axes. Details on the sampled populations, pairwise F_{ST} values and significance levels are provided in Table S9.

initial releases, must be considered in light of confounding factors revealed after the agent had already been widely released: the presence of a cryptic sister species (*M. janthiniformis*) in European field populations of the agent, which was included in shipments of the agent released in North America; and the high level of host fidelity of the two sister species, *M. janthinus* with *L. vulgaris* and *M. janthiniformis* with *L. dalmatica*/*L. genistifolia* (Toševski *et al.*, 2011, 2013).

In 1990, for biological control purposes, the taxonomic status of the agent was traditionally justified and verified by taxonomic specialists (see Jeanneret & Schroeder, 1992). This served to confirm that following the typological species concept, weevils used in pre-release host specificity studies belonged to the unique taxon *M. janthinus*. During the initial period of introduction, the majority of weevils destined for field release in North America were collected from *L. vulgaris* in Europe. However, the introduced weevils exhibited different and unexpected responses to host plants following field release in western North America (AB, BC, ID, MT, OR and WA). Subsequent to an extensive field release programme in North America, a trend became evident in that a rapid expansion of the weevil's populations typically occurred on Dalmatian toadflax, while establishment of *M. janthinus* on yellow toadflax appeared to be marginal (Sing *et al.*, 2005; De Clerck-Floate & McClay, 2013; De Clerck-Floate & Turner, 2013). Moreover, successful establishment and rapid spread of *M. janthiniformis* throughout areas infested with *L. dalmatica* in the past 20 years has been widely correlated with substantial target weed impacts, causing a decline in Dalmatian toadflax plant size and density on a large regional scale (Van Hezewijk *et al.*, 2010). In contrast, establishment and/or spontaneous spreading of *M. janthinus* populations on *L. vulgaris* in North America largely has remained more localised (Sing *et al.*, 2005).

Reasons for the unexpected outcome of the North American toadflax biological control programme, in which *M. janthinus* s.l. had greater success in controlling Dalmatian toadflax than yellow toadflax, were elucidated following integrative taxonomic evaluations of *M. janthinus* populations associated with different host toadflaxes in their native European environment (Toševski *et al.*, 2011). Based on mt*COII* gene analysis and ecological properties, justification was found for promoting two *M.*

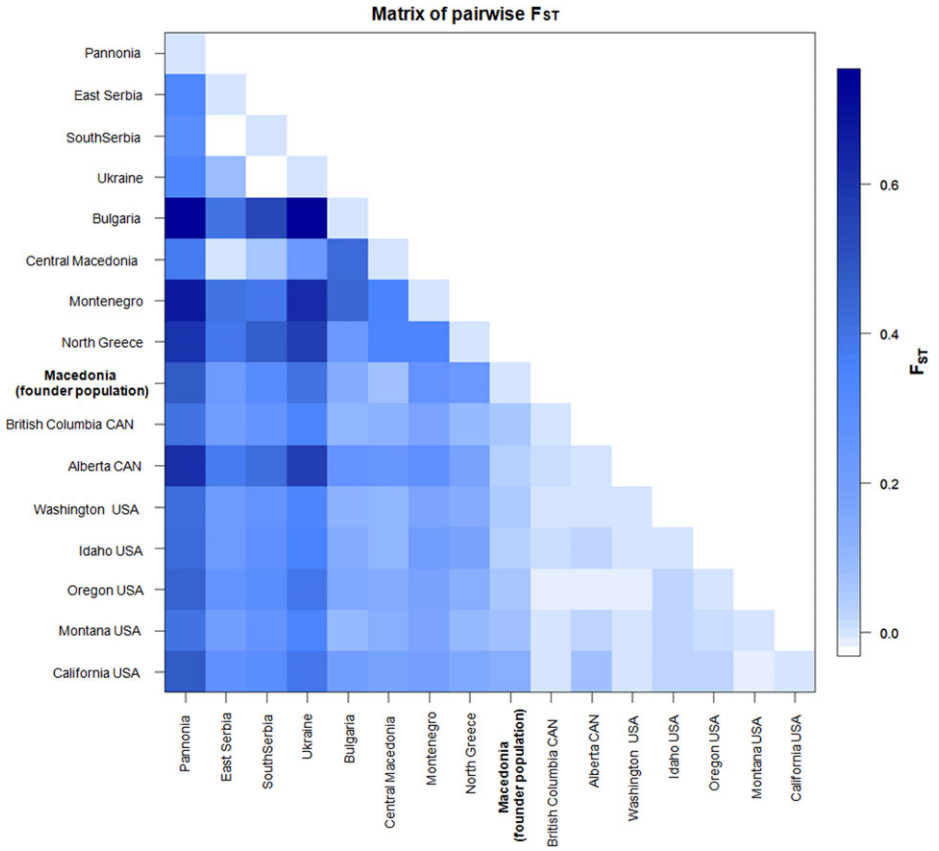

Figure 6 Matrix of pairwise mt*COII*-based F_{ST} for European and North American *Mecinus janthiniformis* populations, sorted by their geographical distribution (regions, provinces or states). Populations are designated on axes. Details on the sampled populations, pairwise F_{ST} values and significance level are provided in Table S10.

janthinus s.l. haplotype groups to distinct species status: *M. janthinus* associated with *L. vulgaris*, and *M. janthiniformis* associated with *L. genistifolia* and *L. dalmatica*. Genetic differentiation was found to be consistent within six sympatric populations, although species were morphologically distinguishable from each other by only a few very subtle characters, which further emphasised their cryptic nature (Toševski *et al.*, 2011).

Our study confirms the origin of *M. janthinus* populations established on yellow toadflax in North America to be southern Germany and northern Switzerland. This finding is in accord with supporting documentation related to 19 shipments of this weevil species to North America between 1991 and 1999. A total of three European haplotypes (v1, v4 and v24) only were confirmed to be present in *M. janthinus* populations now established in North America. Because this represents only 27.3% of the haplotypes registered in the European founder populations, we infer that few founder haplotypes were able to successfully adapt to North American field conditions during the 9-year period when 1585 adults were introduced from Europe. It is worth noting that v1 and v4 were the most frequent haplotypes registered in the Rhine Valley, thus their presence in North America was expected. In addition, three more haplotypes, v1NA, v2NA and v3NA, were recorded only in North American populations of *M. janthinus*. Nevertheless, according to the statistical parsimony network (Fig. 3B), all three North American-only *M. janthinus* haplotypes (v1NA, v2NA and v3NA) are clearly derived from the haplotypes distributed in southern Germany and northern Switzerland. Rareness of v1NA, v2NA and v3NA in the European founder population could explain why we failed to confirm the presence of these haplotypes in native range populations sampled in our study. Such disproportional abundance of the established haplotypes in North America, compared to their infrequency in the European founder populations, emphasises that adaptive capabilities of particular genotypes vary significantly, and are thus not directly dependent on their frequencies in founder populations. However, some widely distributed and frequent native range haplotypes, for example

haplotype v6, were not detected in established North American populations, although recorded as widely present in *M. janthinus* populations throughout Europe, from southern Germany to eastern Serbia (Table S3).

A discrepancy in the distribution of *M. janthinus* and *M. janthiniformis* haplotypes with regard to host plant of origin was recorded at eight of the sampled North American locations (Table S7), a situation to date never observed in their native range (Toševski *et al.,* 2011, 2013). Mixed populations were recorded at three locations solely infested by yellow toadflax (Table S7), Salmon ID, St. Croix NS and Edmonton AB, where the proportion of *M. janthinus* versus *M. janthiniformis* genotypes was 25:8, 8:5 and 27:3, respectively (Tables S5 and S7). In each case where *M. janthiniformis* haplotypes were detected on North American yellow toadflax infestations, their presence corresponded to the prior intentional release of weevils now known to be either *M. janthiniformis*, or mixed populations of *M. janthinus* and *M. janthiniformis*. The presence of genotypes associated with both *M. janthinus* and *M. janthiniformis* at the Salmon and St. Croix sites highlights the ramifications of having both *Mecinus* species present in propagation facility populations prior to field release. Nevertheless, results obtained from the analysis of specimens collected from 51 *L. dalmatica* and 13 *L. vulgaris* locations emphasises the strength of the ecological segregation of *M. janthinus* and *M. janthiniformis* according to host plant of origin, whether in the native range, or following field release in North America (Figs 5 and 6).

When establishment and abundance of *M. janthiniformis* haplotypes recorded in North America is compared with those in the founder populations, the disproportionate abundance of established haplotypes in North America becomes even more pronounced than with *M. janthinus*. Though rare in founder populations, haplotypes gd5, d3, d5, d9 and d14 were detected 3–15 times more frequently in North American populations. In addition, the presence of three haplotypes recorded only in the North American populations of *M. janthiniformis*, d1NA, d2NA and d3NA, suggest that they may have originated under a similar suite of interacting events as discussed above for the *M. janthinus* associated v1NA, v2NA and v3NA haplotypes.

Interestingly, two of these haplotypes (d2NA and d3NA), are registered as less frequent (d2NA), or as very rare (d3NA), recorded in only one out of 35 sequenced adults from the Palisades ID location. The same haplotypes are involved in reticulations in the parsimony network. Reticulate patterns between genotypes could be found in several phylogenetic situations (see Legendre & Makarenkov, 2002) from which patterns of convergent or parallel evolution lead to a homoplasy between haplotypes (Templeton, 1983). Other than independent evolution (convergent or parallel), ancestral state reversal could also lead to homoplasy (Wake *et al.,* 2011). In the case of an introduced population of biocontrol agents, it seems plausible that rare haplotypes evolved in the ancestral range became fixed in the new environment and subsequently lost in the ancestral range, revealing differences in evolutionary patterns presumably due to differences in environmental factors.

According to Fu's *Fs* statistic, which is based on the distribution of haplotypes and is more sensitive to population expansion (Fu, 1997), founder populations of both *Mecinus* species exhibit significant negative values. For species in their native range, this condition is often related to postglacial population expansion (De Jong *et al.,* 2011). Significant negative values for Fu's *Fs* are also exhibited by populations of recently introduced or invasive species (Oduor *et al.,* 2015; Qin *et al.,* 2016); however, in our case, introduced populations of both species revealed non-significant departure from neutrality. These results together with the non-significant values of Tajima's *D* returned for both *Mecinus* species lead us to conclude that these introduced populations are in demographic equilibrium (Excoffier *et al.,* 2005), probably because insufficient time has passed since their North American introduction.

The low level of genetic variation between founder and introduced populations of *M. janthinus* and *M. janthiniformis* confirms the European origin of populations now established in North America. Furthermore, most of the genetic variation can be attributed to individual differences in haplotypes within sampled populations. Population pairwise comparisons also confirm their common origin as introduced populations less differentiated from their respective founder population than from any of the other sampled conspecific populations in Europe. It is also evident from comparisons of pairwise matrices for both species that introduced populations of *M. janthinus* are more differentiated from their founder population than *M. janthiniformis* populations are from their founder population. This strongly suggests that evolutionary pressure involving recognition and acceptance of the appropriate host plant for survival was more expressed in *M. janthinus* than in *M. janthiniformis*, which is in accord with the greater establishment success of the latter species.

All pairwise comparisons between the founder and introduced populations of *M. janthinus* were significant, whereas for *M. janthiniformis* populations, comparing the European founder population with conspecific weevils tested from populations now established in Alberta produced a non-significant value. Significant, although low, differentiation between populations of both species can be attributed to the observed discordance of registered haplotypes and their frequencies in founder and introduced populations. The fact that only three

haplotypes of *M. janthinus* are established in North America of the 14 present in the native range, and nine haplotypes of *M. janthiniformis* are established in North America of the 15 present in the native range, suggests that individual haplotype performance in the new environment is shaping observed discrepancies.

The evolutionary paths of these two weevil species seem to be similar in both their native and introduced environments. Negative values of neutrality tests for European populations of both species and generally positive ones for the respective North American populations can be attributed to differential evolutionary pressures in the native and new environments. The lack of significance could be due to either evolutionary or demographic events in the past, or their reduced effect in the present. Furthermore, as part of independent evolution, individual haplotypes and their differences in performance in the introduced environment need to be taken into account. Mitochondrial haplotypes can exhibit different phenotypic effects, with natural selection in turn acting upon these (Kurbalija Novičić *et al.*, 2015), differential production of reactive oxygen species (Ballard, 2005) or can even differ in adaptive ability in a new environment (Le Roux & Wieczorek, 2009). Therefore, in a diverse founder population, haplotypes could potentially exist that are 'pre-adapted' to cope with environmental challenges encountered *de novo* in the introduced environment (Le Roux & Wieczorek, 2009). Adaptively advantaged haplotypes could successfully establish, propagate and spread in an introduced environment, regardless of their rareness in a founder population. Genetic drift and selection could potentially even enable this type of event. Thus, haplotypes v1NA, v2NA and v3NA of *M. janthinus* and haplotypes d1NA, d2NA and d3NA of *M. janthiniformis* could in fact represent such advantageously 'pre-adapted' haplotypes. Without further research, one can only speculate about the factors that led to the present pattern of haplotype frequency and distribution of both *Mecinus* species in the introduced environment. Nevertheless, our study strongly implies that the behaviour of an introduced biological control agent in a new environment depends on a number of genetic and ecological factors, but with genetic pool as the most important link in the chain of events leading to establishment and later, impact on the targeted weed.

Those receiving and using European shipments of *M. janthinus* s.l. for releases and propagation operated on the premise that they contained one agent species that could be released to biologically control two invasive toadflax species. This misconception proved to be highly consequential, significantly delaying the attainment of consistent and predictable toadflax biological control both in Canada and the USA. One set of actions driven by this assumption was the inadvertent mismatching of *Mecinus* species and targeted *Linaria* during initial releases made in both countries, in many instances resulting in diminished or failed agent establishment. In three of five cases involving the first shipments to Canada of *M. janthinus* collected in the Rhine Valley from *L. vulgaris* (Table S1), post-release monitoring indicates that the weevils did not establish where released on *L. dalmatica*. Five of the six shipments of *M. janthinus* collected from Rhine Valley *L. vulgaris* received by Montana State University during 1996–99 (Table S1) were field released onto Dalmatian toadflax (R.M. Nowierski & S.E. Sing, unpublished data). Similarly, the redistribution of *M. janthiniformis* collected on *L. dalmatica* and released on pure stands of *L. vulgaris* in British Columbia (2) and southern Alberta (2) in 2000 resulted in failed establishment (De Clerck-Floate & McClay, 2013).

Yet another set of actions arising from the incorrect assumption that only one *Mecinus* species was involved was the pooling of weevils obtained from different geographic and/or host origins for propagation and subsequent field releases on either *Linaria* species. The influence of mixed source or mixed species propagation and release on haplotype frequency and distribution of extant North American field populations could not be explicitly traced through the methods used in this study. However, key post-introduction activities (i.e. propagation and redistribution) in addition to ecological filtering (e.g. Richardson & Pyšek, 2012; Gallien & Carboni, 2017) help to explain the array of North American *Mecinus* spp. haplotypes detected. In cases where propagation utilised both *L. vulgaris* and *L. dalmatica* (e.g. Regina SK and Lethbridge AB), it is conceivable that both *Mecinus* species survived on their preferred host until they were field released on the appropriate *Linaria* species, which may explain successful establishments on *L. vulgaris* in Wilbert SK and St. Croix NS, or on *L. dalmatica* in southern Alberta using a pooled *Mecinus* spp. colony from Lethbridge AB.

Another possibility for the co-existence and persistence of both *Mecinus* species genotypes within current North American populations is hybridization. Considering how closely related these two *Mecinus* species are (Caldara *et al.*, 2013), the possibility of hybridization when species are confined together in no-choice conditions, such as in propagation facilities, is conceivable. As a consequence, maternally inherited mitochondrial genotypes of *M. janthinus* within *M. janthiniformis* populations and vice versa, could persist in field populations of both *Mecinus* species. However, over time, their number would be expected to decrease due to further unilateral introgression events with the more abundant parental species, especially in habitats where for both *Mecinus* species only one toadflax host occurs in isolation. This is often the case for the

majority of either *M. janthiniformis* or *M. janthinus* releases in North America.

Apart from putative hybridization between the two weevil species, as discussed above, hybridization between their native range host and another *Linaria* species (Ward *et al.,* 2009) may also play a role in successful colonisation of *Mecinus* spp. populations. DNA sequencing identified two sites where this has occurred in North America, Jaffray BC and Palisades ID. *M. janthiniformis* is established on a hybrid of *L. dalmatica* and *L. vulgaris* (J. Gaskin, personal communication), presumably moving 2.5 km from where they were previously established on the nearest recorded *L. dalmatica* infestation (De Clerck-Floate & McClay, 2013). The Palisades ID hybrid *L. dalmatica* × *L. vulgaris* infestation (Boswell *et al.,* 2016) is extensive and has produced abundant *M. janthiniformis* of diverse haplotypes (Table S6) that have been collected and widely redistributed in Montana and Idaho (J. Milan & S.E. Sing, unpublished data). Because both *Mecinus* species are characterised by their high degree of specificity (Toševski *et al.,* 2011; Caldara *et al.,* 2013) with respect to their host plants of origin, conditions influencing successful establishment and natural distribution during North American field release programmes directly correlated with the obligate utilisation of appropriate host plant, adequate to support unrestricted development of their progeny.

Our study reveals the importance of considering the interacting influences of genetics, evolution, demographic processes and host plant responses to adopted range environmental factors on the behaviour of introduced phytophagous arthropods and consequently, the efficacy of classical biological control programmes. Small introduced populations are influenced by a variety of genetic and demographic processes acting upon them. These include both stochastic and deterministic processes (demographic and environmental stochasticity, Allee effects, genetic drift, inbreeding depression, selection and gene flow) which acting alone or in concert potentially play a crucial role in the establishment of success and efficacy of biological control agents (Fauvergue *et al.,* 2012). Furthermore, genetic drift as the main evolutionary mechanism acting in populations with decreased effective population size, for example bottlenecked populations (Nei *et al.,* 1975), can result in reduced genetic variation, loss of frequent haplotypes or fixation of rare haplotypes (Templeton, 2006).

In the case of *M. janthinus*, during 9 years of introduction, 16 shipments of this agent were sent to propagation facilities in North America. These shipments could have minimised the effect of genetic drift, because, according to Nei *et al.* (1975), little variation will be lost if bottlenecks are of brief duration. However, in propagation facilities, *M. janthinus* populations remained small sized because of predominant rearing on non-preferred host plants. This in turn amplified the influence of ecological factors, involving stochastic processes, but also the influence of co-evolutionary adaptations between highly specialised weevils and their respective host plants on establishment success in the new environment. Interactions of weevils with their host plants (target weeds) following their earliest North American field releases fundamentally influenced the establishment and distribution of *M. janthinus* s.l. in the adopted range. High levels of genetic diversity (Ward *et al.,* 2008) or hybridization (Ward *et al.,* 2009) of target weeds could lead to a situation where weevils are regularly faced with sub-optimal hosts, resulting in a constant search for better hosts. Underperformance has been reported in phytophagous insects dealing with heterogeneous host plant populations (Mopper, 1996) which could also be the case for introduced populations of *M. janthinus* interacting with North American populations of *L. vulgaris*. If North American populations of *L. vulgaris* exhibit different properties than European populations, then the slow establishment of an introduced population of *M. janthinus* could be the result of adaptation to the target weed (Hufbauer & Roderick, 2005).

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 *Mecinus janthinus* s.l. shipments to Canada and the USA, according to 1991–1999 shipping forms retrieved from CABI Europe archives (Switzerland).

Table S2 Molecular identification of dry voucher specimens of *M. janthinus* s.l. originating from shipments, laboratory test populations, propagation facilities and field collection sites.

Table S3 List of *Mecinus janthinus* specimens collected within the species' European distribution used for DNA

extractions. Specimens listed by: haplotype name; DNA extraction code; collection locality name, GPS coordinates, country; host plant association and voucher name. Number in brackets refers to the number of locality as presented in Fig. 1.

Table S4 List of *Mecinus janthiniformis* specimens collected within the species' European distribution used for DNA extractions.

Table S5 List of *Mecinus janthinus* specimens collected within the species' North American distribution used for DNA extractions.

Table S6 List of *Mecinus janthiniformis* specimens collected within the species' North American distribution used for DNA extractions.

Table S7 List of *Mecinus janthinus* and *M. janthiniformis* specimens collected in North America from atypical *Linaria* host (i.e. *M. janthinus* from *L. dalmatica*, *M. janthiniformis* from *L. vulgaris*).

Table S8 AMOVA results on hierarchical distribution of mt*COII* variation within *M. janthinus* and *M. janthiniformis* populations grouped according to geographical distributions in Europe (EU) and North America (NA).

Table S9 Matrix of population pairwise F_{ST} and respective significance levels of 11 *Mecinus janthinus* populations of European (1–7) and North American origin (8–11).

Table S10 Matrix of population pairwise F_{ST} and respective significance levels of 16 *Mecinus janthiniformis* populations of European (1–9) and North American origin (10–16).

Table S11 Comparison of population genetic indices and neutrality tests for European founder populations from southern Macedonia (EU) and introduced North American (NA) populations of *M. janthinus* and *M. janthiniformis*.