Systematic Entomology

Systematic Entomology (2011), DOI: 10.1111/j.1365-3113.2011.00593.x

Morphological, molecular and biological evidence reveal two cryptic species in *Mecinus janthinus* Germar (Coleoptera, Curculionidae), a successful biological control agent of Dalmatian toadflax, *Linaria dalmatica* (Lamiales, Plantaginaceae)

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Abstract. A combined morphological, molecular and biological study shows that the weevil species presently named *Mecinus janthinus* is actually composed of two different cryptic species: *M. janthinus* Germar, 1821 and *M. janthiniformis* Toševski & Caldara **sp.n.** These species are morphologically distinguishable from each other by a few very subtle morphological characters. On the contrary, they are more readily distinguishable by both molecular and biological characters. A molecular assessment based on the mitochondrial DNA cytochrome oxidase subunit II gene revealed fixed differences between the two species with p-distances between samples of both species ranging from 1.3 to 2.4%. In addition to this, the larvae of the two species are found to develop on different species within the genus *Linaria* (Plantaginaceae): *M. janthinus* is associated with yellow toadflax (*L. vulgaris*) and *M. janthiniformis* with broomleaf toadflax (*L. genistifolia*) and Dalmatian toadflax (*L. dalmatica*). Molecular and host use records further suggest the occurrence of a third species associated with *L. vulgaris* within *M. janthinus,* sampled from north Switzerland, central Hungary and east Serbia. The significance of these new findings is of particular importance because species of the *M. janthinus* group are used, or are potential candidates, for the biological control of invasive toadflaxes in North America.

Introduction

Dalmatian toadflax [*Linaria dalmatica* (Linnaeus) Miller], broomleaf toadflax [*L. genistifolia* (Linnaeus) Miller] and yellow toadflax (*L. vulgaris* Miller) (Plantaginaceae) are perennial weeds of European origin that have become naturalized in North America. Dalmatian toadflax was introduced at the end of the 19th century and has spread across every Canadian province and the northern and western U.S.A. (Vujnović & Wein, 1997). Introduced as an ornamental plant in the middle of the 17th century, yellow toadflax is now found in every state in the U.S.A. and across southern Canada. It was recognized as a major problem in parts of the eastern U.S.A. by 1758 (Mack, 2003). There is still much uncertainty regarding toadflax taxonomy, particularly the *L. genistifolia/dalmatica* complex of species and their hybrids. Chater *et al.* (1972)

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Unpublished for the purposes of zoological nomenclature (Art. 8.2, ICZN)

treated *L. dalmatica* as a subspecies of *L. genistifolia*, whereas Hartl (1974) and Davis (1978) treated *L. dalmatica* as a separate species, closely related to *L. genistifolia*. Ward *et al*. (2009) have recently demonstrated that hybridization is occurring between yellow toadflax and Dalmatian toadflax, and that the hybrid progeny are both viable and fertile.

A biological control programme of exotic invasive toadflax species in North America was initiated in 1987 and the first introduction of the stem-mining weevil *Mecinus janthinus* Germar, 1821 was made in 1991 (De Clerck-Floate & Harris, 2002; De Clerck-Floate & Miller, 2002; McClay & De Clerck-Floate, 2002; Sing *et al*., 2005). Population development and the impact of *M. janthinus* in North America have varied widely between different release areas and host plants. During the release programme 1991–1999 in North America, the majority of all weevils originated from *L. vulgaris* in Western Europe (I. Toševski and A. Gassmann, unpublished data), with the exception of about 200 specimens that were released in 1992 from the *L. dalmatica* ssp. *macedonica* (Grisebach) D. A. Sutton collected in Macedonia. The general consensus is that releases in North America led to the rapid establishment of outbreak-level populations on *L. dalmatica*, with a substantial impact on this toadflax (Peterson *et al*., 2005; Wilson *et al*., 2005; Van Hezewijk *et al*., 2010). In contrast, only rare and low-density populations were reported on *L. vulgaris* in Canada (McClay & Hughes, 2007; R. De Clerck-Floate, personal communication) and the U.S.A. (S. Sing, personal communication), some 20 years after the introduction. The limited establishment of *M. janthinus* on *L. vulgaris* in North America may be correlated with high levels of genetic diversity within and among invasive yellow toadflax populations in North America recently reported by Ward *et al*. (2008). However, the limited establishment of the introductions on *L. vulgaris* and contrasting success on *L. dalmatica* raise the question of to what extent *M. janthinus* may be in need of taxonomic revision.

Mecinus janthinus (Curculioninae, Mecinini) is characterized mainly by a very long body and the blue coloration of the dorsal integument. It differs from other *Mecinus* species with similar body shape and vestiture colour (e.g. *M. heydenii* Wencker, 1866 and related species) in the form of the rostrum, which is distinctly less curved in a lateral view (Hoffmann, 1958; Smreczyński, 1976; Lohse & Tischler, 1983). Probably as a consequence of its characteristic phenotype, the taxonomic position of *M. janthinus* is not burdened with many uncertainties of synonymy. Smreczyński (1976) placed *M. pillichi* Endrödi, 1969, described from a unique male collected in western Hungary, in synonymy with *M. janthinus*. The only species that seems to be very closely related to *M. janthinus* is *M. kaemmereri* Wagner, 1927, a species that has been described from specimens collected from *Linaria purpurea* (Linnaeus) Miller in Sicily.

Prerelease host specificity tests carried out with a population of *M. janthinus* collected from the *L. genistifolia*/*dalmatica* plant complex in Macedonia revealed that this population would only develop on a few related *Linaria* species (Jeanneret & Schroeder, 1991). Comparative host acceptance tests with Canadian *L. vulgaris* and *L. dalmatica* and European *L. vulgaris* showed no significant difference in the number of eggs laid in no-choice conditions. However, the percentage of larvae completing development on *L. vulgaris* was 8.3%. Unfortunately, larval development on *L. dalmatica* was not studied (Jeanneret & Schroeder, 1992). Additional postrelease host suitability tests carried out with *M. janthinus* sampled from *L. genistifolia* in Serbia revealed that only 21.5% of the larvae completed development on *L. vulgaris* of Serbian origin, whereas no larval development was recorded on *L. vulgaris* originating from North America. In contrast, 86.3% of the larvae of *M. janthinus* from *L. vulgaris* in Serbia completed development on this host plant (Toševski *et al*., 2007). In a study of the development rate of *M. janthinus* on potted North American *L. vulgaris* and *L. dalmatica*, McClay & Hughes (2007) recorded a very low survival of the weevil on *L. dalmatica*, but the origin of the weevils used in their study was not given. These authors observed that eggs laid in *L. dalmatica* were often surrounded by a dense layer of hard, waterlogged stem tissue that produced a visible swelling in the plant stem, whereas this response was never seen in *L. vulgaris*. Similar stem reactions have been observed with the weevils *Rhinusa pilosa* (Gyllenhal, 1838) and *M. heydenii* (both associated with *L. vulgaris*) after being exposed to Dalmatian toadflax for oviposition (Toševski *et al*., 2005, 2007).

The series of larval development studies described above strongly suggest the possible occurrence of cryptic species with different biological properties, host preferences and induced responses from the target hosts. In the past two decades an increasing number of phylogenetic, biodiversity and conservation studies, based on the rapid development of molecular methods, have led to the discovery of numerous cryptic species (e.g. Hebert *et al*., 2004; Pons *et al*., 2006; Pfenninger & Schwenk, 2007; Burns *et al*., 2008). However, the clarification of nomenclature issues and stabilization of the status of the existing synonyms, which are related to a particular cryptic species complex, are usually not part of molecular-based studies (see Hebert *et al*., 2004; Burns *et al*., 2007). As a result, newly discovered species are often not properly described and/or are usually signed alphabetically with capitalization of the voucher labels. Here we present the results of morphological, biological and genetic studies for *M. janthinus* associated with toadflaxes in Europe in an attempt to better understand the current taxonomy of this variably successful biological control agent of invasive toadflaxes in North America.

Material and methods

Acronyms

The following acronyms for public (curators in parentheses) and private collections are used:

BMNH, Department of Entomology, Natural History Museum, London, U.K. (Max Barclay)

DEI, Deutsches Entomologisches Institut, Müncheberg, Germany (Lutz Behne)
GOC, Giuseppe Osella Collection, Verona, Italy
HNHM, Hungarian Natural History Museum, Budapest, Hungary (Otto Merkl)
ITC, Ivo Toševski Collection, Belgrade, Serbia
JKC, Jiri Krátký Collection, Hradec Králové, Czech Republic
MKC, Michael Košt'ál Collection, Brno, Czech Republic
MLUH, Institut für Zoologie, Martin-Luther-Universität, Halle, Germany (Karla Schneider)
MNHN, Muséum National d'Histoire Naturelle, Paris, France (Hélène Perrin)
NHMB, Naturhistorisches Museum, Basel, Switzerland (Eva Sprecher)
RCC, Roberto Caldara Collection, Milano, Italy
ZIN, Zoological Institute, Russian Academy of Sciences, St Petersburg, Russia (Boris A. Korotyaev)

Specimens examined

This study was based on the examination of type specimens of *M. janthinus* (MLUH), *M. pillichi* (HNHM) and *M. kaemmereri* (NHMB), specimens from several private and public collections and newly collected material. Over 2000 specimens were examined in order to determine the geographical distribution of the studied taxa.

Morphological study

As possible diagnostic characters we considered body size (rostrum excluded), shape of head, eye, antennae, rostrum, prothorax, elytra and abdomen; sculpture and vestiture of rostrum, pronotum, elytra and abdomen; shape of male (aedeagus, tegmen, spiculum gastrale) and female (spermatheca, ovipositor, spiculum ventrale) terminalia.

Host suitability study

Specimens associated with *L. vulgaris* were collected near Mihajlovac (Negotin, east Serbia) and those associated with *L. genistifolia* near Preševo (Vranje, south Serbia). At both sites, the sympatric occurrence of the two toadflax species was excluded at a distance of at least 500 m. Weevils were sampled in early spring at the beginning of their emergence in the field. No-choice oviposition and larval development tests were set up on 8 April 2010 and the control and test plants were exposed to a single mated female until mid-June. In addition, three females were set up as a no food control to determine the maximum starvation period. A total of 12 mated females from *L. vulgaris* and 13 mated females from *L. genistifolia* were used in this study. During testing females were transferred to fresh plants approximately every 7–9 days. Female longevity (days) was recorded until mid-June. All plants were dissected for larval, pupal and adult development on 20 July 2010. The significance of results in the larval development test was assessed with an ANOVA test.

Material for DNA study

Specimens were collected from within the broad distribution of *M. janthinus* in central and southeast Europe (Fig. 1, Appendix S1), including freshly collected specimens from the type localities to evaluate their taxonomic status with regard to known synonyms of *M. janthinus*. Four specimens were analysed from the type locality of Germar's *M. janthinus* (Odenbach, Germany) and four specimens from the type locality of *M. pillichi* (Simontornya, Hungary). To unequivocally determine host-plant affiliation, particularly where *L. vulgaris* and plants from the *L. genistifolia/dalmatica* complex of species occurred sympatrically, 27 weevils were reared from their original field host plants (12 ex *L. vulgaris* and 15 ex *L. genistifolia*). Eight specimens of *M. kaemmereri* were collected from *L. pupurea* in Sicily. In addition, three specimens were collected on *L. genistifolia* ssp. *confertiflora* (Boissier) Davis and two specimens on *L. corifolia* Desfontaines from west and northeast Turkey. A total of 192 specimens were collected for genetic analyses. All specimens were preserved in 96% ethanol and stored at −20°C until DNA extraction. After DNA extraction, weevil adults were prepared as voucher dry specimens for morphological study.

DNA extraction

Individual weevils or larvae were punctured on the ventrolateral side of the second thoracic segment and total DNA was extracted using QIAGEN Dneasy® Blood & Tissue Kit (Germany) according to the manufacturer's instructions. The same procedure was used to extract DNA from four dry syntype specimens of *M. janthinus* deposed in Germar's collection at MLUH and Endrödi's holotype specimen of *M. pillichi* deposed at HNHM.

Polymerase chain reaction (PCR) amplification and sequencing

The mitochondrial cytochrome oxidase subunit II gene (COII) is a powerful marker for the discrimination of evolutionary divergence of host-plant choice for oviposition because of its female inheritance combined with its high mutation rate and small effective population size (0.25) relative to the nuclear genome. It has proven to be an appropriate marker in previous studies investigating differentiation within species and the determination of host-plant affiliation within related species (e.g. Caldara *et al*., 2008; Hernández-Vera *et al*., 2010). From newly collected material, the complete COII gene was amplified using the primers TL2-J-3038 (5′-TAATATGGCAGATTAGTGCATTGGA-3′) (Emerson *et al*., 2000) and TK-N 3782 (5′-GAGACCATTACTTGCTTTCAGT

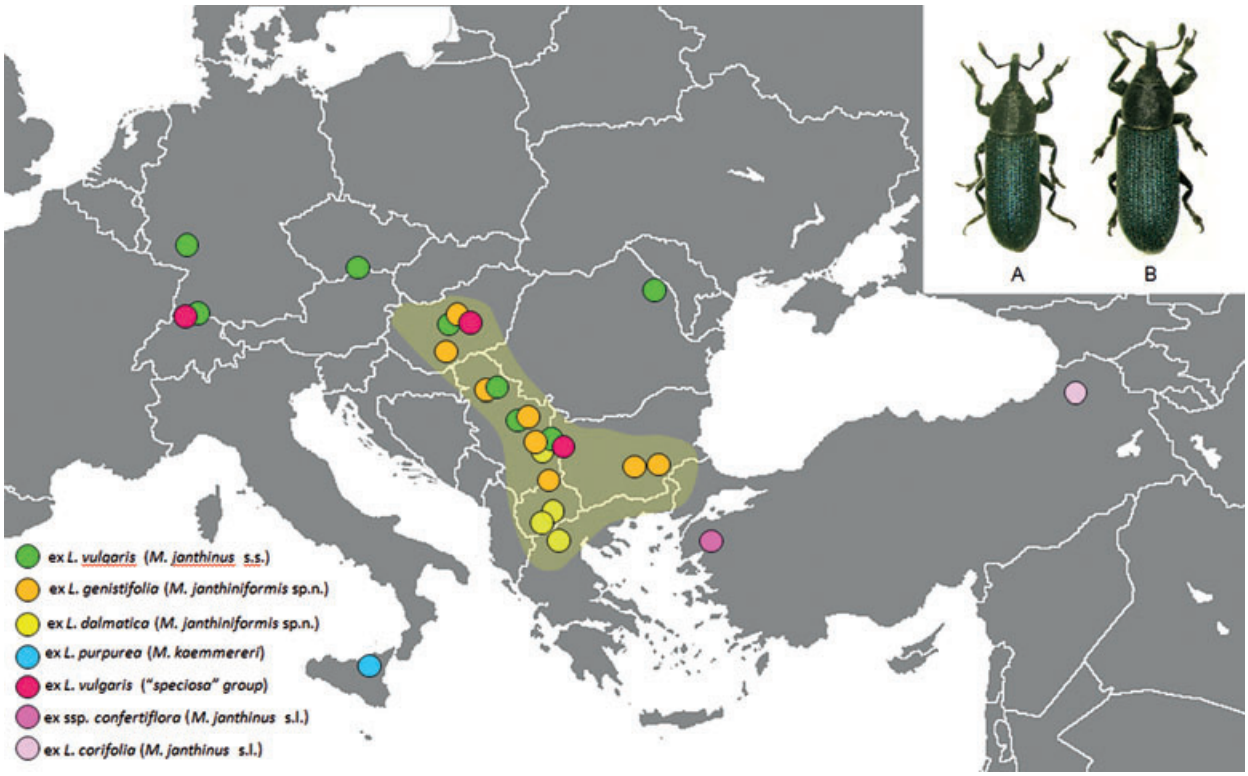

Fig. 1. Sampling sites and associated host plants for weevils from the *Mecinus janthinus* s.l. complex (putative distribution range of *M. janthiniformis* **sp.n.** is shaded yellow); (A) habitus of *M. janthinus* Germar; (B) habitus of *M. janthiniformis* **sp.n.**

CATCT-3′) (Harrison Laboratory, Cornell University, Ithaca, NY, U.S.A.). PCR contained NH4 buffer (1×), 5 mM $MgCl_2$, 0.8 mM of each dNTP, 0.75 μM of each primer and 0.75 U of *Taq* polymerase (Fermentas, Lithuania) in a 20 μL final volume. PCR cycles were carried out in a Mastercycler ep gradient S (Eppendorf, Germany) applying the following thermal steps: 95°C for 5 min (initial denaturation), 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), and sequenced on automated equipment by BMR Service (Padova, Italy). For most specimens, sequences of 698 bp were obtained with the forward primer only, whereas for several specimens sequencing was performed with both primers to obtain sequences of full-length PCR products (784 bp).

For dry syntype specimens, a set of three primer pairs (Table 1) was designed for specific amplification of short mitochondrial COII fragments (less than 100 bp in length), considering that mitochondrial DNA (mtDNA) in dry insect material is frequently highly degraded and fragmented. Primers designed for the amplification of these short mtDNA fragments were positioned within the COII gene sequence so they would amplify regions of the gene determined to be informative and specific for host-plant association of the *M. janthinus* s.l. specimens at positions 171, 174, 252 and 489 of the COII gene. These 'host plant specific' nucleotides within the COII gene, targeted for sequencing in type series specimens, were determined after alignment of all COII sequences obtained from newly collected specimens of *M. janthinus* s.l. associated with *L. vulgaris* and the *L. genistifolia/dalmatica* plant complex (Appendix S1). Designed primers were tailed with 18 bp long M13 sequences – M13REV (5′-CAGGAAACAGCTATGACC-3′) for all forward primers and M13(21) (5′-TGTAAAACGACGG CCAGT-3′) for all reverse primers. These oligonucleotides were used to improve sequence read length, by sequencing with the adaptor only. 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 extension time, which was reduced to 30 s, with the final extension time reduced to 3 min. PCR products of short fragments were cleaned as described above and sequenced with a reverse or forward tail adaptor as indicated in Table 1.

Evolutionary tree construction and haplotype network construction

Sequences were edited with FINCHTV v.1.4.0 (www.geospiza.com) and aligned with CLUSTALW integrated in MEGA4 (Tamura *et al.*, 2007). Aligned sequences of all unique haplotypes were used in the final phylogenetic analyses. A maximum parsimony phylogeny was reconstructed

Table 1. Primers used for the amplification of short mitochondrial cytochrome oxidase subunit II (COII) fragments.

Primer[a]	Primer sequence (without tails)	Amplicon length (without tails) (bp)	Sequencing primer
171F	5′ GGACAAATATTGCTATCTA 3′	83	Tail M13REV
171R	5′ GTTCATACTATCTCAATTAATTG 3′		
252F	5′ ATTGCCCTTCCATCACTACGA 3′	88	Tail M13(21)
252R	5′ ATCATTGGTGACCAATTGTT 3′		
489F	5′ GATAACCGAATAATTATCCCA 3′	94	Tail M13REV
489R	5′ ATTCCTAGAGATGGAATTGT 3′		

[a]Primer name following position of informative nucleotide related to complete COII gene length in *Mecinus janthinus* (1–678 nt).

with MEGA5 (Tamura *et al*., 2011) using the close neighbour interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (50 replicates). Five hundred bootstrap replicates were performed to assess branch support in the resulting tree topology. The tree was rooted using *Gymnetron rotundicolle* Gyllenhal, 1838 and *Rhinusa antirrhini* (Paykull, 1800) as outgroups (GenBank accession numbers JN167166 and JN167167). Inter- and intraspecific uncorrected pairwise genetic distances between haplotypes with different host affiliation were calculated in MEGA5. Although evolutionary gene trees may be informative at the intraspecific level, phenomena such as the persistence of ancestral haplotypes mean that such data are frequently better visualized in reticulated graphs or networks (Posada & Crandall, 2001). Thus, TCS version 1.21 (Clement *et al*., 2000) was also employed to infer haplotype networks using statistical parsimony (Templeton *et al*., 1992) with a confidence limit of 95%.

Results

Morphological study

Body size was the character that differed most significantly ($P < 0.01$) between specimens associated with *L. vulgaris* and those associated with the *L. genistifolia/dalmatica* plant complex: body length (rostrum excluded) was 3.5 ± 0.3 mm (range 2.3–4.0 mm, $n = 73$) and 4.1 ± 0.4 mm (range 3.2–6.0 mm, $n = 64$), respectively. Taking into account that body size can be influenced by many biotic and abiotic factors (plant condition and food quality, competition with other larvae for food resource, temperature regime during larval development, etc.) it cannot be used as a reliable taxonomic character per se, but it is a useful tool for a reasonable presumption of the weevil host-plant affiliation. Although we found no diagnostic morphometric differences, some morphological characters useful in the separation of approximately 70–80% of specimens collected either on *L. vulgaris* or the *L. genistifolia/dalmatica* plant complex were the curvature of the rostrum in the female in lateral view, the sculpture of the pronotum, the vestiture of the elytral interstriae, the shape of the median lobe of the aedeagus in its distal part. This last character was the most consistent differential character between 20 specimens associated with *L. vulgaris* from Germany (four), Hungary (eight) and east Serbia (eight) and 20 specimens associated with the *L. genistifolia/dalmatica* plant complex from Hungary (four), east Serbia (eight) and Macedonia (eight). All other characters, such as the shape of the head, eye, antennae, prothorax, elytra and abdomen, were not suitable to distinguish specimens that use different *Linaria* species as the host plant, confirming the cryptic nature of weevils from the *M. janthinus* species complex.

Host specificity study

Offspring survival rates were similar across both host-plant groups when individuals were reared on the host-plant species they were sampled from (88.1% on *L. vulgaris* and 90.8% on *L. genistifolia*). Offspring survival rate was much lower when individuals were raised on the alternative host (17.3% on *L. genistifolia* and 7.8% on *L. vulgaris*) (Table 2). No females lived longer than 10 days in the no plant control.

Mitochondrial COII analyses

The final alignment of COII sequences consisted of 678 bp with a total of 54 (8%) polymorphic sites, of which 35 were parsimony informative. Forty-four different haplotypes (Appendix S2) were obtained across 192 sequenced specimens, and these are available from GenBank under accession numbers JN037471–JN037662 (Appendix S1). In total, 15 haplotypes are associated with *L. vulgaris* as a host plant, 19 with the *L. genistifolia/dalmatica* plant complex, three with *L. purpurea* from Sicily, two with *L. genistifolia* ssp. *confertiflora* from west Turkey and two with *L. corifolia* from northeast Turkey. In addition, three haplotypes (here referred to '*speciosa*' group sequences) were identified occurring sympatrically with populations of *M. janthinus* on *L. vulgaris* from Switzerland, Hungary and Serbia.

The average pairwise genetic distance (p-distance) across all sequences was 1.5%. The average pairwise sequence divergence was between 0.1 and 0.5% within plant groups and between 1.0 and 2.9% between plant groups (Table 3). The maximum genetic divergence of 3.2% was recorded between haplotypes associated with *L. vulgaris* and those of the '*speciosa*' group also associated with *L. vulgaris* at Basel in Switzerland, Pincehely in central Hungary and Kalna in east Serbia. Pairwise sequence divergence between haplotypes

Table 2. Female longevity, no-choice oviposition and larval development tests with *Mecinus janthinus* s.l. originating from *Linaria vulgaris* and *M. janthiniformis* **sp.n.** from *L. genistifolia.*

Plant species	No. of females tested	No. of plants tested	Mean females longevity, days ± SD (range)	Total no. live larvae, pupae and adults	Total no. dead larvae, pupae and adults	Total no. registered develop-ments	Survival rate (%)
			Mecinus janthinus s.s.				
No plant control	3	—	8 ± 1.7 (range 7–10)	—	—	—	—
L. vulgaris (control)	4	45	58.2 ± 9.0 (range 45–64)	118	16	134	88.8
L. genistifolia	5	30	61 ± 8.0 (range 53–70)	18	86	104	17.3
			Mecinus janthiniformis **sp.n.**				
No plant control	3	—	9.3 ± 0.6 (range 9–10)	—	—	—	—
L. genistifolia (control)	5	44	53 ± 9.2 (range 43–68)	218	22	240	90.8%
L. vulgaris	5	24	27.8 ± 3.7 (range 23–32)	4	47	51	7.8

Table 3. Average mitochondrial DNA cytochrome oxidase subunit II divergence based on the pairwise analysis (p-distance method) between all *Mecinus janthinus* s.l. haplotypes, grouped according to their host-plant affiliation.

			P (SE)					
Sequences group	d_1 (SE)	d_2 (SE)	1	2	3	4	5	6
1. ex *L. vulgaris*	0.015 (0.003)	0.005 (0.001)	—	0.003	0.006	0.005	0.005	0.005
2. ex *L. purpurea*		0.002 (0.001)	0.010	—	0.006	0.005	0.005	0.005
3. '*speciosa*' group ex *L. vulgaris*		0.002 (0.001)	0.029	0.025	—	0.004	0.005	0.005
4. ex *L. g. confertiflora*		0.001 (0.001)	0.020	0.016	0.013	—	0.003	0.004
5. ex *L. corifolia*		0.003 (0.002)	0.024	0.020	0.018	0.010	—	0.004
6. ex *L. genistifolia/dalmatica*		0.005 (0.001)	0.019	0.018	0.023	0.015	0.19	—

d_1, divergence over all sequence pairs; d_2, divergence over sequence pairs within groups; P, p-distance over sequence pairs between groups; SE, standard error.

associated with *L. vulgaris* and those associated with the *L. genistifolia/dalmatica* plant complex ranged between 1.3 and 2.4%, and ranged between 0.6 and 1.3% between haplotypes associated with *L. vulgaris* and *L. purpurea*.

Evolutionary relationships inferred using maximum parsimony revealed two distinct mitochondrial lineages associated with *L. vulgaris* and *L. genistifolia/dalmatica*, with bootstrap support values higher than 60%, whereas the 'speciosa' haplotypes clustered together within haplotypes from *L. genistifolia* ssp. *confertiflora* (west Turkey) and *L. corifolia* from northeast Turkey (Fig. 2). The haplotype network constructed using statistical parsimony with TCS v.1.21 (Clement *et al*., 2000) revealed no ambiguous connections between mitochondrial lineages, with the exception of a single reticulation within the '*speciosa*' group, and confirmed clustering of haplotypes according to their host affiliation, clearly assigning populations associated with *L. vulgaris* and with *L. genistifolia/dalmatica* (Fig. 3).

Short mitochondrial fragment analysis of type material

An alignment of 169 COII gene sequences from specimens associated with *L. vulgaris* and the *L. genistifolia/dalmatica* complex revealed a total of eight nucleotide positions to be informative for the specific identification of individuals developing on each host plant or plant complex (Table 4). Primers were designed to capture a subset of this variation for nucleotides at positions 171($G_{vulg.}$–$A_{genist.}$), 174 ($M_{vulg.}$–$T_{genist.}$), 252 ($G_{vulg.}$–$A_{genist.}$) and 489 ($A_{vulg.}$–$G_{genist.}$). These primers were used for the determination of the host-type affiliation of the type specimens. Short mitochondrial fragments were successfully amplified with all three primer pairs for all four syntypes of *M. janthinus* and the holotype of *M. pillichi*, enabling determination of nucleotide states at the four diagnostic sites (Table 4). Sequence data from syntypes (Table 4) revealed that two of the four specimens from the Germar's type series (a male 2.3 mm and a female 3.0 mm long) belong to the species that develops on *L. vulgaris*, whereas the other two females (both 3.7 mm long) belong to the species that develops on the *L. genistifolia/dalmatica* plant complex. Sequence data from the *M. pillichi* sample revealed this specimen to also have originated from *L. vulgaris*. Thus, this name should be treated as a younger synonym of *M. janthinus* as proposed by Smreczyński (1976).

Taxonomy

According to morphological, molecular and biological data, at least two taxa can be distinguished within the *M. janthinus* species complex: *M. janthinus*, which is associated with *L. vulgaris*, and the *Mecinus* species, which is associated with *L. genistifolia* and *L. dalmatica* (*L. genistifolia/dalmatica*

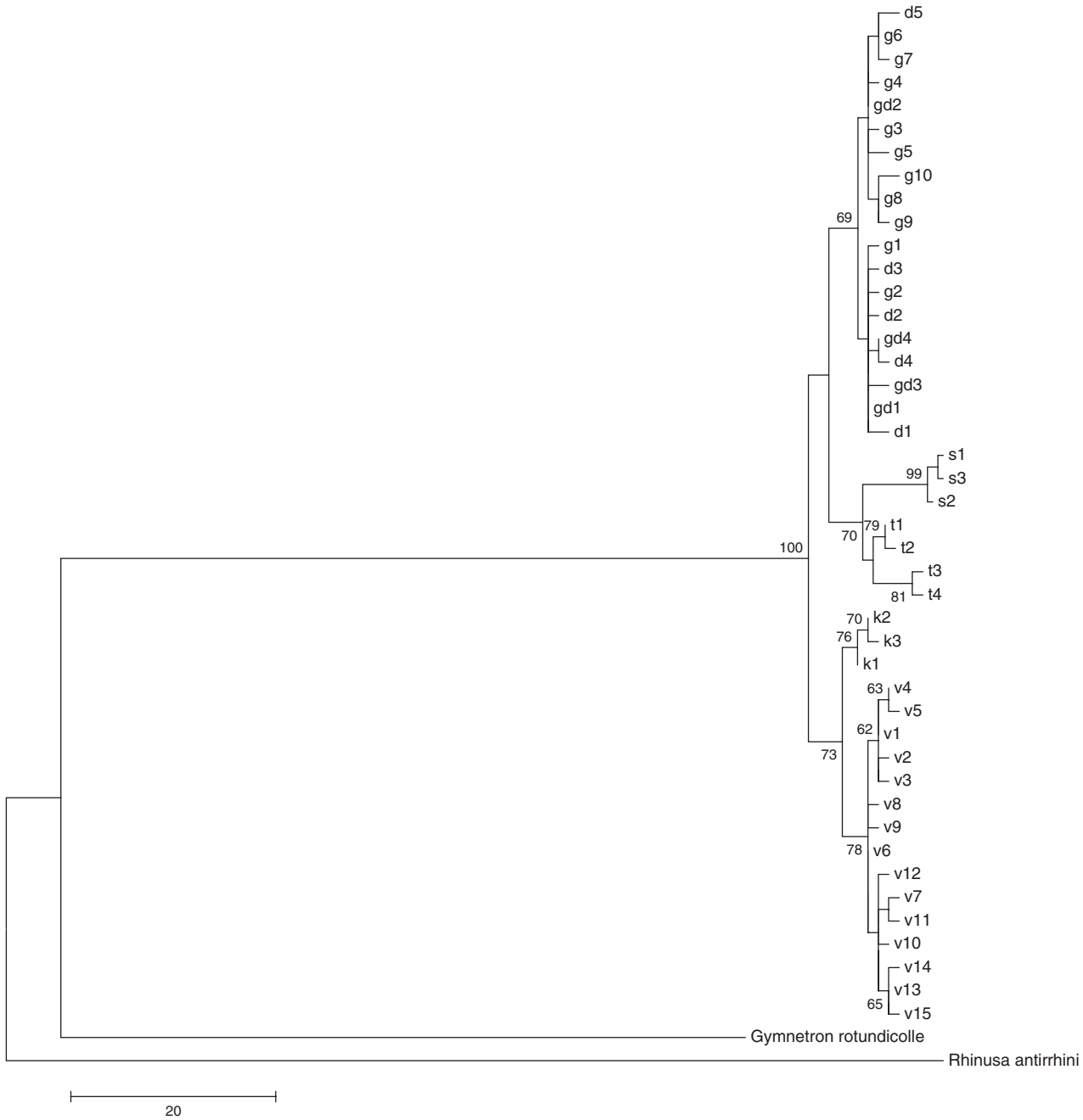

Fig. 2. One of 288 most-parsimonious phylogenetic trees inferred from 678 bp of the mitochondrial DNA COII gene for 44 *Mecinus janthinus* s.l. haplotypes sampled from different host plants (length = 312, consistency index = 0.74, retention index = 0.89). The percentages of replicate trees in which associated taxa clustered together from a bootstrap analysis (500 replicates) are indicated (values lower than 60% are omitted).

plant complex). The taxonomic position of *M. kaemmereri,* which is associated with *L. purpurea*, is still uncertain. It differs from *M. janthinus* by its larger body size and the longer and less sculptured rostrum of the female. The close phylogenetic relationship with the *M. janthinus* haplotype group (Fig. 3) and its distinct geographical location (southern Italy and Sicily) suggest differentiation at least at a subspecific level. The lack of abundant material for a genetic study and insufficient information on the biology and ecology of the specimens associated with *L. corifolia* and *L. genistifolia* ssp. *confertiflora* from Turkey require us to leave these populations temporarily unclassified. For similar reasons, the taxonomic position of the '*speciosa*' group also remains an open issue.

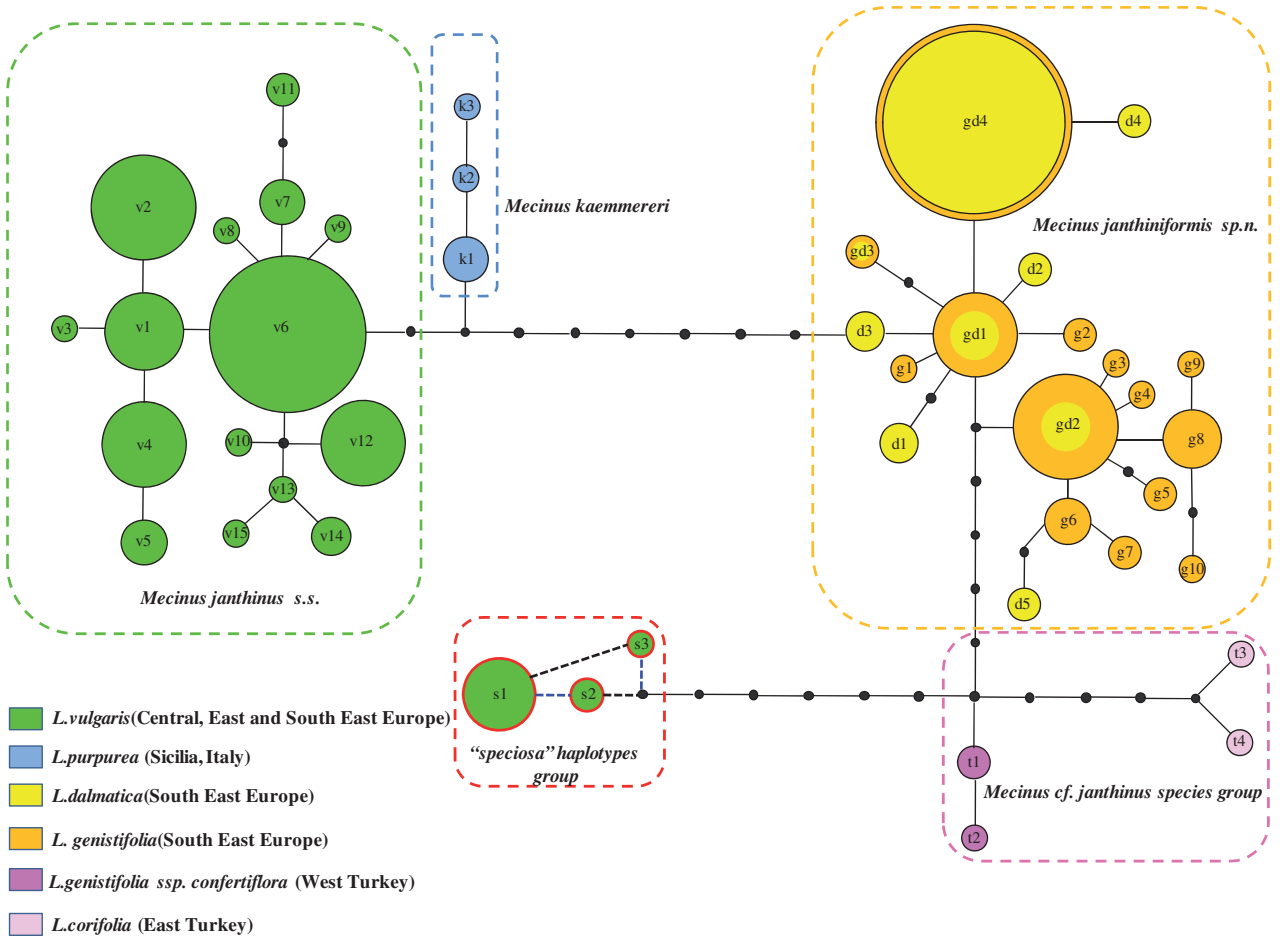

Fig. 3. Statistical parsimony haplotype network constructed from 678 bp of the mitochondrial DNA COII gene for *Mecinus janthinus* s.l. Each line connecting circles is one mutational difference. Numbered circles represent sampled haplotypes, the size being proportional to frequency. Small black circles represent missing or unsampled haplotypes.

Mecinus janthinus Germar

(Figs 1A, 4A)

Mecinus janthinus Germar, 1821: 319. Lectotype ♂ by present designation, Germany: Odenbach (MLUH); Rösenschöld (1838: 779); Reitter (1908: 8); Hustache (1931: 404), Hoffmann (1958: 1269), Smreczyński (1976: 24), Lohse & Tischler (1983: 261), Arzanov (2000: 1086).

Mecinus pillichi Endrödi, 1969: 276. Holotype ♂, Hungary: Simontornya; Smreczyński (1976: 24).

Type material examined. Mecinus janthinus was described based on specimens from Odenbach (Rhineland-Palatinate, Germany). In Germar's collection at MLUH under the name *janthinus* we found four specimens without labels but on

Table 4. Interspecific informative sites between *Mecinus janthinus* s.l. specimens associated with *Linaria vulgaris* and *L. genistifolia/dalmatica*.

		Informative sites within the COII gene							
M. janthinus s.l. specimens	Number of specimens sequenced	65	114	171[a]	174[a]	202	252[a]	489[a]	522
ex L. vulgaris	84	T	C	G	M	T	G	A	C
ex L. genistifolia/dalmatica	85	A	T	A	T	C	A	G	T
M. janthinus (Lectotype)	1	—	—	G	C	—	G	A	—
M. janthinus (Paralectotype)	1	—	—	G	C	—	G	A	—
M. janthinus (Germar's syntypes 1, 4)	2	—	—	A	T	—	A	G	—
M. pillichi (Holotype)	1	—	—	G	C	—	G	A	—

[a] Site positions used to distinguish host affiliation of Museum's type material.
COII, cytochrome oxidase subunit II.

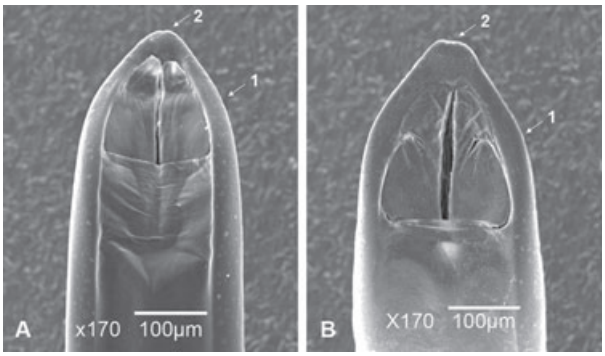

Fig. 4. Distal part of aedeagus in dorsal view of: (A) *Mecinus janthinus* Germar; (B) *M. janthiniformis* **sp.n.** Arrows indicate the differences between the two species in the curvature of the sides of the apical portion (arrow 1) and the shape of the tip (arrow 2).

the whole well preserved and corresponding well to the original description: one very small male (2.3 mm) glued onto a small triangular card and one female (3.0 mm) glued onto a small subquadrate card, both pinned on the same pin; two large females (3.7 mm) both pinned at the base of the right elytron. Analyses based on short COII gene fragments show that two specimens from the type series (the male and the female 3.0 mm long) belong to the species that develops on *L. vulgaris*, whereas the two larger females belong to the species living on the *L. genistifolia/dalmatica* complex. Considering that the type locality of *M. janthinus* is Odenbach (Rhineland-Palatinate, Germany) and given that plants from the *L. genistifolia/dalmatica* complex are not native in this region, we have decided to designate the male specimen as the lectotype of *M. janthinus*, which now bears a red label with 'Lectotype ♂, *Mecinus janthinus* Germar, 1821, DNA code IT-869/2010; des. Caldara & Toševski 2010'. The second specimen, a 3.0 mm long female, was designated as 'Paralectotype ♀, *Mecinus janthinus* Germar, 1821, DNA code IT-870/2010; des. Caldara & Toševski 2010'. The other two specimens were excluded from the type series. In addition, four mtCOII gene sequences of *M. janthinus* from Odenbach (type locality) are available from GenBank under accession numbers JN037476–JN037479 (Appendix S1).

Mecinus pillichi, which has been described from a single male specimen from Simontornya (Hungary) and deposited at HNMH, had already been dissected when examined by us. Endrödi (1969) reported that this specimen differs from other blue coloured species of *Mecinus* by the elytral striae formed by very broad punctures. We agree with Smreczyński (1976) who synonymized this taxon with *M. janthinus*. The study of mtDNA COII sequence data reinforces our conclusion that Endrödi's specimen is associated with *L. vulgaris* and thus being synonymic with *M. janthinus*. Four mtDNA COII gene sequences of *M. janthinus* from Simontornya (type locality of *M. pillichi*) are available from GenBank under accession numbers JN037524–JN037527 (Appendix S1).

Other material examined. In addition to the type specimens we also examined approximately 2000 specimens from localities across the entire distribution of *M. janthinus* and deposited in public and private collections (DEI; GOC; HNHM; JKC; MKC; MNHN; ZIN), as well as newly collected adults from 18 localities in the European distribution area of this species. Moreover, we examined all specimens reared from *L. vulgaris*, which were used for genetic analyses as presented in Appendix S1.

Diagnosis. Length 2.3–4.0 mm (rostrum excluded). Mean body length for males 3.3 ± 0.3 mm (2.3–4.0 mm, $n = 33$) and for females 3.6 ± 0.3 mm (2.9–4.0 mm, $n = 40$). Integument black in colour except pronotum and elytra dark blue with metallic reflections. Rostrum in lateral view moderately and regularly curved, moderately long in male, somewhat longer in female (male 0.80–0.88×, female 1.03–1.09× as long as pronotum length), in male moderately striate-punctured to apex, in female only in basal half, then almost smooth. Pronotum sculpture formed by deep punctures regular in shape and size, intervals between punctures narrow, smooth and shining, clearly visible between sparse seta-like long white scales, widest at basal third. Elytra very long, about twice as long as width, with interstriae roughly sculptured and covered with recumbent seta-like white scales almost completely arranged in a single median row. Profemora with distinct sharp tooth in male, unarmed in female. Aedeagus (Fig. 4A) with sides parallel, gradually narrowing in distal third (arrow 1), toward apex ending in form of subacute tip (arrow 2).

Molecular diagnosis. Using molecular biology tools, *M. janthinus* can be reliably and precisely identified by possessing a specific combination of nucleotides in the complete sequence of COII gene at site 65 (T), 114 (C), 171 (G), 174 (M), 202 (T), 252 (G), 489 (A) and 522 (C).

Distribution. Northern (Sweden, Baltic States), central and southeastern Europe (Great Britain, France, Belgium, Holland, Poland, Germany, Switzerland, Italy, Czech Republic, Slovakia, Hungary, Austria, Serbia, Ukraine, Moldova Republic, Romania, Bulgaria, Greece), Russia from the western borders (north to south) to southern central Siberia, the Caucasian states, Turkey. The species has been introduced in North America for biological control of toadflaxes in 1991–1999 (Wilson *et al*., 2005).

Biology. The host plant of *M. janthinus* is *L. vulgaris*. It is a univoltine species, which is usually found in lowlands and hilly slopes up to 500 m altitude. The adults overwinter in the stems of the host plant, inside an elongated pupal chamber built by the last instar larva prior to pupation. The adults emerge from the dry stems in early March and feed intensively on the newly growing shoots of the host plant. Copulation occurs shortly after and the egg-laying period lasts from the end of March until mid-June. Oviposition occurs on actively

growing shoots and the preferred oviposition site is always the widest part of the stem, usually within the lower stem. Before oviposition, the female prepares a shallow hole in which the egg is deposited. During oviposition, the female secretes a sticky fluid that fixes the egg to the plant tissue. Females lay one, rarely two, eggs per shoot. *Mecinus janthinus* is a true stem borer with larvae feeding and mining in the central part of the stem. Mine length typically varies from 1 to 2 cm, but can be up to 10 cm long if the stem tissue is stressed by a low water regime. Under these conditions, larvae move to the upper or lower stem where tissue conditions provide a better source of nutrition. In laboratory conditions, embryogenesis lasts from 6 to 7 days and complete larval development takes between 23 and 34 days. Development from egg to pupation takes between 50 and 63 days (Janneret & Schroeder, 1992). In southeast Europe, adults of *M. janthinus* can occasionally be collected in early spring on *L. genistifolia*, *L. rubioides* ssp. *nissana* and *L. angustissima* at stands where these plants are sympatric with *L. vulgaris*, but larval development has never been recorded on these plant species (I. Toševski, unpublished data). The dominant parasitoid of *M. janthinus* in southeast Europe is *Entedon sparetus* Walker, 1839 (Hymenoptera, Eulophidae, Entedoninae), which parasite over 50% of larvae.

Mecinus janthiniformis **Toševski & Caldara sp.n.**
(Figs 1B, 4B)
Holotype, ♂, Macedonia: Streževo, ex larva, ex *L. dalmatica* ssp. *macedonica*, N41 04.205 E21 11.430, 890 m 23 August 2009, lgt. I.Toševski, DNA-IT544, voucher mksd23, haplotype gd4, (BMNH).
Paratypes and depositories are listed in Appendix S3.

Etymology. The name refers to the close similarity in the shape with *M. janthinus*.

Diagnosis. Morphology as in *M. janthinus,* except body generally larger (4.1 ± 0.4 mm; range 3.2–6.0 mm); mean body length for males 4.1 ± 0.3 mm (3.2–4.7 mm) and for females 4.1 ± 0.5 mm (3.3–6.0 mm); apical portion of the rostrum in female in lateral view more curved; punctures of pronotum slightly smaller, more densely adpressed; scales of elytral interstriae denser, arranged in two rows on part of several interstriae; aedeagus (Fig. 4B) with sides slightly more abruptly narrowed in subapical part (arrow 1), towards apex ending in form of subtruncate tip (arrow 2).

Molecular diagnosis. Mecinus janthiniformis can be identified by possessing a specific combination of eight nucleotides in the complete sequence of COII gene at the site 65 (A), 114 (T), 171 (A), 174 (T), 202 (C), 252 (A), 489 (G) and 522 (T).

Distribution. Eastern part of central and southeastern Europe (Hungary, Serbia, Romania, Bulgaria, Macedonia and Greece), but probably widely distributed throughout the native range of its host plant *L. genistifolia* (Ukraine, Moldova Republic and from the western borders of south Russia to southern central Siberia and the northern Caucasian states) and *L. dalmatica* (southeast Europe). A small number of adults have been collected on *L. dalmatica* in Macedonia and introduced to North America in 1992–1993 as *M. janthinus* (I. Toševski and A. Gassmann, unpublished data; Wilson *et al*., 2005).

Biology. The host plants of *M. janthiniformis* are *L. genistifolia* and *L. dalmatica*, as well as all variable forms between these two plant species. The species is frequently present in host-plant stands from lowlands to mountain pastures and meadows up to 1500 m altitude. Like *M. janthinus*, it is a univoltine species that overwinters at the adult stage inside the stem of the host plant. Adults emerge in early April and feed intensively on the apical leaves and the apical part of new shoots. Copulation takes place in April and first oviposition can be observed at the beginning of May. The egg-laying period lasts from May until mid-July. In contrast to *M. janthinus*, *M. janthiniformis* females oviposit either on the upper part of the central stem, or on the basal part of lateral flowering branches. As a result of oviposition and larval development, the plant induces a slightly elongated gall-like alternation in which larval development will take place inside a relatively short larval chamber (about 1 cm long). During development, larvae continuously feed on the gall-like tissue from the chamber walls. Similar to *M. janthinus*, *M. janthiniformis* is heavily parasitized by the endoparasitic wasp *Entedon sparetus* (Eulophidae), which can reduce population densities of this weevil by more than 80%.

Discussion

Analyses of mitochondrial COII gene sequences reveal relatively low but clear and consistent host-associated genetic structure among sampled populations of *M. janthinus* s.l. from different host-plant taxa. In both maximum parsimony and statistical parsimony analyses, weevil COII haplotypes retrieved from a particular host-plant taxon cluster together. Two haplotype groups are assigned full species status: *M. janthinus* is associated with yellow toadflax (*L. vulgaris*) and *M. janthiniformis* with broomleaf and Dalmatian toadflaxes (*L. genistifolia* and *L. dalmatica*). The two *Mecinus* species are morphologically distinguishable from each other by only a few very subtle characters, but genetic differentiation was consistent within six sympatric populations of *L. vulgaris* and *L. genistifolia* (one in Hungary and five in Serbia, see Appendix S1). Additionally, conclusions of host plant-associated genetic differentiation are supported by substantially higher offspring survival of the two *Mecinus* species on their respective host-plant species. Other biological characteristics also distinguish the two species. The larvae of *M. janthinus* mine the lower stem of *L. vulgaris*. In contrast, larvae of *M. janthiniformis* develop in the upper parts of the main stem and very often inside lateral branches of *L. genistifolia* or *L. dalmatica*, causing well-developed semi-gall enlargement. We have observed (I. Toševski and A. Gassmann, unpublished

data) that *M. janthinus* appears in the field about 1 month earlier than *M. janthiniformis*, but weevil activity overlaps at least 3 months within the sympatric stands of the host-plant taxa (i.e. until mid-June for *M. janthinus* and until mid-July for *M. janthiniformis*). Thus, there is no evidence for temporal or spatial isolation of the two weevil species, which strongly suggests that ecological differentiation between the two species is a key factor underlying their reproductive isolation.

The taxonomic position and status of *M. kaemmereri* associated with *L. purpurea* from southern Italy and Sicily remain unclear. This species is genetically closely related to *M. janthinus*, although morphologically it seems more similar to *M. janthiniformis*. However, further and more detailed biological studies are needed to resolve its taxonomic status. Also, no conclusion can be made for the specimens associated with *L. genistifolia* ssp. *confertifora* and *L. corifolia* from Turkey because of the small number of specimens analysed. Thus, we treat these specimens as still 'taxonomically unclassified', but as a part of the *M. janthinus* s.l. complex.

The discovery of the '*speciosa*' group of sequences within nominal *M. janthinus* populations from very distant sites in central and southeast Europe is of particular interest. MtDNA COII sequences are most closely related to the *M. janthiniformis* group sequences, but because the host plant is *L. vulgaris*, the possible existence of a third species in the *M. janthinus* species complex must be considered. A total of seven of 33 specimens (21%) collected from Basel (north Switzerland) yielded '*speciosa*' group sequences, and this was one of the main collection sites for *M. janthinus* during the release programme for North America. All three haplotypes from distant sites in Europe share only single nucleotide substitution from each other. The close relationship of the '*speciosa*' group with *M. janthinus* s.l. specimens associated with *L. genistifolia* ssp. *confertiflora* and *L. corifolia* from Turkey may suggest that the '*speciosa*' group represents mitochondrial remains or traces of a relict species, which was widely distributed in the West Palearctic and which presently persists and inhabits different toadflax species in middle Asia. One of the possible scenarios could be that the '*speciosa*' group results from a host shift on *L. vulgaris* following the extinction of its original host plant in Europe. In this case, the '*speciosa*' sequences should be treated as a relict of an unknown *Mecinus* species whose populations are in the process of extinction. However, it may also be possible that the patterns of haplotype relationships are due to incomplete lineage sorting. To evaluate possible evolutionary significance of the '*speciosa*' group at this stage is not possible without the study of additional specimens (e.g. from Italy) and the analysis of nuclear genes to test for linkage disequilibrium with mtDNA divergences in sympatry (e.g. Cicconardi *et al*., 2010).

Taken together, our data argue for a role for ecological divergence, with different host-plant resource use being a driving force for genetic differentiation within the *M. janthinus* s.l. complex. Cryptic speciation has already been reported for weevils within the tribe Mecinini associated with toadflaxes. Phylogenetic analyses of mtDNA COII sequences within the *Rhinusa antirrhini* species complex reveal clear genetic structure with six mitochondrial lineages associated with different taxa of the genus *Linaria* (Hernández-Vera *et al*., 2010). This is a striking result because the external morphology of *R. antirrhini* associated with *L. vulgaris* and the *L. genistifolia/dalmatica* complex are practically undistinguishable, despite genetic divergence for the mtDNA COII gene exceeding 14% (p-distance uncorrected). Similar results with high mitochondrial divergence were obtained with weevils from the *R. antirrhini* species complex associated to *L. rubioides*, *L. genistifolia* ssp. *confertiflora*, *L. genistifolia* ssp. *linifolia* and *L. genistifolia* ssp. *artvinensis* (Hernández-Vera *et al*., 2010). In addition, high mitochondrial divergence has also been reported between the cryptic weevil species *R. pilosa* and *R. brondelii*, both stem gall inducers on *L. vulgaris* and *L. genistifolia*, respectively (Caldara *et al*., 2008). Substantial divergence at the mtDNA COII gene has also been described within another cryptic complex of *Mecinus* weevil species from European toadflaxes for *M. heydenii* associated with *L. vulgaris* and *L. genistifolia*, respectively (Toševski *et al*., 2008).

Considering recently acquired genetic data, it appears that host-plant effects play a significant role in weevil species differentiation within toadflaxes (Hernández-Vera *et al*., 2010). Ecological speciation driven by host-plant selection is not limited to populations of *M. janthinus* s.l. only, but seems to apply to several other species that are using *L. vulgaris* and *L. genistifolia* as host plants (Caldara *et al*., 2008). Host-plant choice by ovipositing females, which probably plays a crucial role in the identification of a suitable host for successful offspring development, is faithfully captured by divergent mitochondrial lineages. Strong host-plant defence reactions recorded in cross no-choice oviposition experiments and larval development tests (Toševski *et al*., 2007), greatly support this hypothesis and suggest the existence of highly specific adaptations and co-evolutionary relationships between weevil taxa associated with different toadflaxes. As such, it now seems clear that the success of a highly specific biological control agent, such as *M. janthinus* s.l., will be affected by its limited capacity to utilize nonhost toadflax species that may also occur in areas of release in North America.

Conclusion

In the early 1990s, the typological species concept was used to select appropriate specimens of *M. janthinus* for introduction into North America for a release programme for the control of toadflaxes. It had been considered that the host range of *M. janthinus* included both *L. vulgaris* in central Europe, but also *L. vulgaris* and *L. genistifolia* s.l. in southeast Europe. The existence of at least two cryptic species associated with *L. vulgaris* and the *L. genistifolia/dalmatica* species complex in Europe with distinct biological characteristics would appear to at least partly explain both the successes and failures of biological control of toadflaxes in North America. An ongoing study to determine the taxonomic status and European origin of *M. janthinus* s.l. in North America is complemented by a study of the genetic variability of yellow and Dalmatian toadflaxes in

Europe and in North America (I. Toševski and A. Gassmann, unpublished data).

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-3113.2011.00593.x

Appendix S1. List of specimens used in this study, sorted by species name, host-plant affiliation, locality and haplotype name.

Appendix S2. *Mecinus janthinus* species complex: haplotype diversity, frequencies and geographical distribution.

Appendix S3. *Mecinus janthiniformis*, list of paratypes and depositories.

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Acknowledgements

We thank the Wyoming Biological Control Steering Committee, the Ministry of Forests and Range, British Columbia Provincial Government, USDA-APHIS-CPHST, USDA Forest Service through the Montana State University, and the California Department of Food and Agriculture who supported this programme in 2008, 2009 and 2010. We gratefully acknowledge the support of Dr De Clerck-Floate (AAFC, Lethbridge, Canada) and Dr Andrew Norton (Colorado State University, U.S.A.), the co-ordinators of the toadflax consortium in North America. This research was partly funded by Consejo Nacional de Ciencia y Tecnologia (CONACYT) Mexico, and in part by grant III43001 (Ministry of Education and Science of the Republic of Serbia) within the framework of a biological control programme against exotic toadflaxes in North America.

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Accepted 23 June 2011