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PCR-RFLP-based method for reliable discrimination of cryptic species within *Mecinus janthinus* species complex (Mecinini, Curculionidae) introduced in North America for biological control of invasive toadflaxes

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Abstract Several populations of the stem-mining weevil *Mecinus janthinus* Germar species complex (Mecinini, Curculionidae), identified based on morphological characteristics, have been introduced in North America for the biological control of invasive toadflaxes of European origin: *Linaria vulgaris* Miller and *L. dalmatica* (L.) Miller (Plantaginaceae). According to the mitochondrial cytochrome oxidase subunit II (COII) gene haplotype divergence of *Mecinus janthinus* species complex, a total of 20 *M. janthinus s.s.*, 3 *M. janthinus s.l.* of the 'speciosa' genotype and 29 *M. janthiniformis* haplotypes have been recorded across their native range in central and southeastern Europe. A polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) diagnostic assay of COII gene using *Hpy*188III and *Mnl*I enzyme-mix, was developed for fast and cost-effective discrimination of these morphologically very similar cryptic weevil species. It is shown that digestion generates unique 4-fragment restriction profile in *M. janthinus s.s.*, 2-fragment profile in *M. janthiniformis* and 3-fragment profile in *M. janthinus s.l.* 'speciosa' group of haplotypes, allowing precise identification of each species or genotype. The proposed method represents a practical tool for fast and accurate identification of the target biocontrol agents and should prevent using inappropriate weevil species in redistribution programs for biological control of invasive toadflax species.

Keywords Curculionidae · Invasive toadflaxes · *Mecinus janthiniformis* · *Mecinus janthinus* · Molecular identification of biocontrol agent · PCR-RFLP diagnostic assay

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Introduction

Broad-leaved Dalmatian toadflax [*Linaria dalmatica* (L.) Miller], narrow-leaved Dalmatian toadflax [*L. genistifolia* (L.) Miller] and yellow toadflax (*L. vulgaris* Miller) (Plantaginaceae) are perennial weeds of European origin that have become naturalized in North America (Sutton 1988). Introduced as an ornamental in the middle of the 17th century, yellow toadflax is now found in every state in the USA and across southern Canada (Mack 2003). Dalmatian toadflax was introduced at the end of the 19th century and has spread across every Canadian province and the northern and western USA (Vujnović and Wein 1997) where it has become a major problem on dry, rocky or gravelly soils. There is still much uncertainty

regarding toadflax taxonomy, particularly the *L. genistifolia/dalmatica* complex of species and their hybrids. Chater et al. (1972) treated *Linaria dalmatica* as a subspecies of *L. genistifolia*. Hartl (1974) and Davis (1978) treated *L. dalmatica* as a separate species closely related to *L. genistifolia*, while Sutton (1988) resumed Davis's statement and comments that morphological types called *L. dalmatica* may belong to various hybrid forms between *L. genistifolia* and *L. grandiflora* Desf. More recently, Niketić and Tomović (2008) identified 36 names used in the literature for *L. genistifolia* taxa and Ward et al. (2009) demonstrated that hybridization is occurring between yellow toadflax and Dalmatian toadflax in North America, and that the hybrid progeny is viable and fertile. The uncertainty about the taxonomy of invasive toadflaxes in North America and their ability to hybridize makes the selection of effective biocontrol agents a challenging task, in particular when they prove to be highly host specific.

A biological control program for alien invasive toadflax species in North America was initiated in 1987 and the first introduction of the stem-mining weevil *Mecinus janthinus* Germar was made in 1991 (De Clerck-Floate and Harris 2002; McClay and De Clerck-Floate 2002). Population development and the impact of *M. janthinus* in North America have varied widely between different release areas and host plants. Although the great majority of all weevils released in North America originated from *L. vulgaris,* collected in South Germany and North Switzerland (Toševski and Gassmann, unpublished data), the general consensus is that releases on *L. dalmatica* in North America led to a rapid buildup of outbreak-level populations of *M. janthinus*, with substantial impact on this weed (Peterson et al. 2005; Wilson et al. 2005; van Hezewijk et al. 2010; Schat et al. 2011). In contrast, only scarce and low-density populations of this weevil were recently reported on yellow toadflax in Canada (McClay and Hughes 2007; De Clerck-Floate, personal communication) and USA (Sing, personal communication).

The need to revise the taxonomical status of *M. janthinus* associated with *Linaria* spp. in Europe and to determine the exact European origin of the successful weevil populations used as biological control agents against invasive toadflaxes in North America increased after reports on high levels of genetic diversity within and among invasive yellow and Dalmatian toadflax populations in North America (Ward et al. 2008, 2009). A genetic study of European *M. janthinus (s.l.)* populations using mithochondrial DNA sequences, revealed extensive genetic divergence between populations associated with different *Linaria* spp. and conspicuous clustering by their host-plant affiliations (Toševski et al. 2011). In both maximum parsimony and statistical parsimony analyses, weevil COII haplotypes retrieved from a particular host-plant taxon cluster together. Two haplotype groups were assigned full species status: *M. janthinus* is associated with yellow toadflax and *M. janthiniformis* Toševski and Caldara with broad-leaved and narrow-leaved Dalmatian toadflaxes. In addition, three haplotypes (referred to as 'speciosa' group sequences) were identified occurring sympatrically with populations of *M. janthinus* on *L. vulgaris* populations from very distant sites in central and southeast Europe.

The possible existence of two cryptic weevil species within the populations of *M. janthinus* introduced in North America raised the question of how to distinguish those species in redistribution programs in order to improve their selection and effectiveness as biological control agents. Restriction fragment length polymorphism (RFLP) is a powerful tool which provides relatively simple and precise method when applied for species identification, especially if it is applied on DNA products obtained by polymerase chain reaction (PCR) (Gaskin et al. 2011). In general, molecular methods are still not routine in biological control programs and for this reason there are only few studies in the literature that have characterized genetic variation within or among populations of weed biocontrol agents (Rauth and Hufbauer 2009). In contrast, PCR-RFLP-based methods are in wide use as a diagnostic tool for economically important or quarantine pests (Scheffer et al. 2001; Brunner et al. 2002; Salazar et al. 2002; Barr et al. 2006; McKern and Szalanski 2007). In the case of cryptic species, PCR-RFLP should be the method of choice since it is inexpensive, simple, reliable, and repeatable and can be used on the insect during any developmental stage (Gaskin et al. 2011). Here we report the development of a PCR-RFLP-based diagnostic tool to differentiate between *Mecinus* weevils which are being used for the biological control of toadflaxes in North America.

Materials and methods

Material for DNA study

For this study we used previously reported DNA material of *M. janthinus s.l.* (Toševski et al. 2011) as well as newly collected specimens (Table 1). From the previously reported material we used the DNA of selected specimens representing each of the formerly identified haplotypes upon the mtDNA cytochrome oxidase subunit II (COII) gene sequences of *M. janthinus s.l.* collected in central and southeastern Europe (Table 2). Template DNA of these specimens was used for de novo PCR amplification of the COII gene and submitted to RFLP analyses, along with newly collected specimens.

Between 2011 and 2012, we conducted additional sampling of *M. janthinus* and *M. janthiniformis* in order to get a better overview of the haplotype diversity and frequency in the native range of both weevil species (Table 1). With this sampling we attempted to increase the number of haplotypes for further analyses and cover majority of the possible haplotypes that could be expected in the introduced environment of North America. A total of 137 specimens were collected in the following European countries: Switzerland, northern Italy, eastern Serbia, Montenegro, northeastern Bulgaria, southern Macedonia and northern Greece (Table 1). Newly collected weevil specimens were kept in 96 % ethanol and stored at −20 °C until DNA extraction. Individual weevils were punctured on the ventro-lateral side of the second thoracic segment and total DNA was extracted using DNeasy® Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions.

PCR amplification and sequencing

The mitochondrial COII gene was chosen as a marker for the identification of host-plant associated species or genotype within the *M. janthinus* species complex. This gene has been previously proven to be an appropriate marker to determine host plant affiliation within related species from the tribe Mecinini (Caldara et al. 2008; Hernández-Vera et al. 2010) and was used for genetic differentiation of cryptic species within the *M. janthinus* species complex (Toševski et al. 2011).

MtDNA fragments including the complete COII gene were amplified using TL2-J-3038 (5′-TAATATGGCA GATTAGTGCATTGGA-3′) (Emerson et al. 2000) and TK-N-3782 (5′-GAGACCATTACTTGCTTCAGTC TATCT-3′) (Harrison Laboratory, Cornell University, Ithaca, NY, USA) primers located in the adjacent tRNA genes. Amplification reactions were performed in a 20-µl final reaction volume containing Kapabiosystems high yield reaction buffer A with Mg (1×), 3.5 mM $MgCl_2$, 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. PCR cycles were carried out in a Mastercycler ep gradient S (Eppendorf) applying the following thermal steps: initial denaturation for 5 min at 95 °C followed by 40 cycles of denaturation step at 95 °C for 1 min, annealing at 45 °C for 1 min and elongation step at 72 °C for 2 min. Final elongation was performed at 72 °C for 10 min.

The PCR amplicons were purified using the QIAquick PCR purification Kit (QIAGEN, Hilden, Germany) and sequenced on automated equipment by BMR Service (Padova, Italy) or Macrogen Inc. (Seoul, South Korea) using one, or both primers used for amplification. For most of the analyzed specimens sequences of full length COII gene (678-bp) were obtained with the forward primer only, while for a number of specimens, reading was done with both primers in order to obtain sequences of full length PCR products (784-bp). The obtained sequences were edited using FinchTV v.1.4.0 (http://www.geospiza.com) and aligned using ClustalW program integrated into MEGA5 software (Tamura et al. 2011).

Sequences of *M. janthinus s.l.* COII haplotypes detected in this study for the first time were deposited in the GenBank database under the accession numbers JX631141-55. Accession numbers of the complete COII gene sequences of all recorded haplotypes are presented in Table 2.

Virtual restriction analysis and gel plotting

In silico restriction analysis was performed for all registered COII gene haplotypes to identify suitable restriction enzymes that could be used for species and 'speciosa' genotype differentiation within the *M. janthinus* species complex. Sequences of all haplotypes were aligned and presence of species-specific or 'speciosa' genotype-specific SNPs (single nucleotide polymorphisms) in the recognition sites for restriction endonucleases were determined using the pDRAW32 software (AcaClone Software, http://www.acaclone.com). Full-length sequences, corresponding to the exact size of TL2-J-3038/TK-N3782 PCR fragment, were exported to the pDRAW32 program and virtually

Table 1 List of specimens analyzed within the survey performed to track diverse haplotypes of *M. janthinus* and *M. janthiniformis* in their native range

Country	Location	Host plant	No. of specimens/ haplotypes[a]	Newly detected haplotypes[b]
Mecinus janthinus				
Germany	Weil am Rhein	*L. vulgaris*	2/2	–
	Odenbach	*L. vulgaris*	1/1	–
Switzerland	Basel (railway station)	*L. vulgaris*	2/2	–
	Domont, Delémont	*L. vulgaris*	5/5	v17, v19
	Delémont	*L. vulgaris*	4/2	v18
Italy	Vidigulfo, Pavia	*L. vulgaris*	2/2	–
	Campogaliano, Modena	*L. vulgaris*	2/1	v20
Serbia	Kalna	*L. vulgaris*	4/3	v16
Mecinus janthiniformis				
Serbia	Kalna	*L. genistifolia* ssp. *genistifolia*	5/2	–
Bulgaria	South Mt Rila	*L. genistifolia* ssp. *sofiana*	2/1	–
	Popovica, Plovdiv	*L. genistifolia* ssp. *sofiana*	1/1	–
	Elhovo	*L. genistifolia* ssp. *genistifolia*	3/1	–
	Primorsko	*L. genistifolia* ssp. *genistifolia*	3/2	g11
	Jasnaja Poljana, Primorsko	*L. genistifolia* ssp. *genistifolia*	10/2	g13
	Arkutino	*L. genistifolia* ssp. *genistifolia*	3/1	–
	Harmanli	*L. genistifolia* ssp. *sofiana*	4/2	g12
Macedonia	Demir Kapija	*L. genistifolia* ssp. *genistifolia*	1/1	–
	Prilep	*L. dalmatica* ssp. *macedonica*	7/3	–
	Pretor	*L. dalmatica* ssp. *macedonica*	3/3	–
Greece	Florina	*L. dalmatica* ssp. *macedonica*	44/9	d8, d9, d10, d11
	Sotiris	*L. dalmatica* ssp. *macedonica*	7/4	d9
	Limnotopos	*L. genistifolia* ssp. *genistifolia*	2/2	d12
	Dispilio, Kastoria	*L. dalmatica* ssp. *macedonica*	9/2	d8
	Kastoria	*L. dalmatica* ssp. *macedonica*	8/3	d8
Montenegro	Medun	*L. dalmatica* ssp. *dalmatica*	3/2	d6, d7

[a] Number of analyzed specimens per locality/number of different detected haplotypes

[b] Newly detected haplotypes compared to Toševski et al. (2011)

digested with the selected restrictive enzymes using the option 'explicitly select' for single-enzyme digestion or 'enzyme-mix' for double-enzyme digestion and separated on a 1.5 % agarose gel. Virtual restriction patterns were compared with actual enzymatic RFLP patterns of amplicons obtained from specimens representing each COII haplotype.

RFLP analysis

Based on the putative restriction map of the *M. janthinus* species complex COII gene haplotypes, *Hpy*188III and *Mnl*I endonucleases were used for the in vitro digestion. TL2-J-3038/TK-N3782 PCR amplified DNA fragments of all 52 specimens representing unique haplotypes (Table 2) were digested with both enzymes in reactions with a single enzyme and in a reaction with mix of both enzymes. All digestion reactions were performed at 37 °C for 16 h using 1× NEBuffer 4, according to manufacturer's instructions (New England BioLabs, Inc., USA). Restriction products were separated by electrophoresis on 13 % polyacrylamide gels in TBE buffer (Tris–Borate 90 mM, EDTA 1 mM), stained with ethidium bromide and visualised with a UV transilluminator.

Table 2 List of mtCOII accession sequence codes of specimens used in the RFLP study, sorted by species name, host plant affiliation, locality and haplotype

Accession sequence code	Host plant	Location/country	GPS coordinate	Haplotype[a] (frequency)	*Hpy*188III/*Mnl*I RFLP profile[b]
Mecinus janthinus					
JN037471*	*L. vulgaris*	Basel (railway station), Switzerland	N47 34.977 E07 36.137	v1 (12)	4-fragment
JN037480*	*L. vulgaris*	Weil am Rhein, Germany	N47 35.389 E07 36.498	v2 (14)	4-fragment
JN037494*	*L. vulgaris*	Weil am Rhein, Germany	N47 35.389 E07 36.498	v3 (3)	4-fragment
JN037495*	*L. vulgaris*	Weil am Rhein, Germany	N47 35.389 E07 36.498	v4 (13)	4-fragment
JN037506*	*L. vulgaris*	Weil am Rhein, Germany	N47 35.389 E07 36.498	v5 (6)	4-fragment
JN037510*	*L. vulgaris*	Basel, (railway station), Switzerland	N47 34.977 E07 36.137	v6 (23)	4-fragment
JN037531*	*L. vulgaris*	Blace, Central Serbia	N43 14.772 E21 15.514	v7 (5)	4-fragment
JN037535*	*L. vulgaris*	Pincehely, Hungary	N46 40.565 E18 24.177	v8 (1)	4-fragment
JN037536*	*L. vulgaris*	Znojmo, Czechia	N48 49.510 E16 02.740	v9 (1)	4-fragment
JN037537*	*L. vulgaris*	Popricani, Romania	N47 16.414 E27 29.839	v10 (1)	4-fragment
JN037538*	*L. vulgaris*	Tamnič, East Serbia	N44 06.038 E22 30.045	v11 (3)	4-fragment
JN037540*	*L. vulgaris*	Tamnič, East Serbia	N44 06.038 E22 30.045	v12 (10)	4-fragment
JN037550*	*L. vulgaris*	Negotin, East Serbia	N44 16.610 E22 30.480	v13 (1)	4-fragment
JN037551*	*L. vulgaris*	Tamnič, East Serbia	N44 06.038 E22 30.045	v14 (3)	4-fragment
JN037554*	*L. vulgaris*	Negotin, East Serbia	N44 16.610 E22 30.480	v15 (1)	4-fragment
JX631141	*L. vulgaris*	Kalna, East Serbia	N43 29.450 E22 19.712	v16 (2)	4-fragment
JX631142	*L. vulgaris*	Domont, Switzerland	N47 22.130 E07 18.601	v17 (1)	4-fragment
JX631143	*L. vulgaris*	Delémont, Switzerland	N47 22.380 E07 19.452	v18 (1)	4-fragment
JX631144	*L. vulgaris*	Delémont, Switzerland	N47 22.380 E07 19.452	v19 (3)	4-fragment
JX631145	*L. vulgaris*	Modena, Italy	N44 40.470 E10 50.270	v20 (2)	4-fragment
M. janthinus s.l. 'speciosa' genotype					
JN037555*	*L. vulgaris*	Basel (railway station), Switzerland	N47 34.977 E07 36.137	s1 (7)	3-fragment
JN037562*	*L. vulgaris*	Kalna, East Serbia	N43 29.450 E22 19.712	s2 (2)	3-fragment
JN037564*	*L. vulgaris*	Pincehely, Hungary	N46 40.565 E18 24.177	s3 (1)	3-fragment
M. janthiniformis					
JN037578*	*L. genist.* ssp. *sofiana*	Harmanli, East Bulgaria	N41 56.438 E25 52.066	gd1 (41)	2-fragment
JN037587*	*L. genist.* ssp. *genistifolia*	Gradec, South Macedonia	N41 23.836 E22 18.836	gd2 (24)	2-fragment
JN037601*	*L. genist.* ssp. *genistifolia*	Preševo, South Serbia	N42 15.663 E21 42.100	gd3 (5)	2-fragment
JN037603*	*L. dalm.* ssp. *macedonica*	Pretor, South Macedonia	N41 03.780 E21 03.118	gd4 (38)	2-fragment

Table 2 continued

Accession sequence code	Host plant	Location/country	GPS coordinate	Haplotype[a] (frequency)	*Hpy*188III/*Mnl*I RFLP profile[b]
JN037630*	*L. dalm.* ssp. *macedonica*	Sotiras, North Greece	N40 40.050 E21 40.050	d1 (17)	2-fragment
JN037633*	*L. dalm.* ssp. *macedonica*	Pretor, South Macedonia	N41 03.780 E21 03.118	d2 (3)	2-fragment
JN037635*	*L. dalm.* ssp. *macedonica*	Drenovska Gorge, South Macedonia	N41 26.153 E21 49.092	d3 (3)	2-fragment
JN037638*	*L. dalm.* ssp. *macedonica*	Pretor, South Macedonia	N41 03.780 E21 03.118	d4 (2)	2-fragment
JN037640*	*L. dalm.* ssp. *macedonica*	Ohrid-Prespa, South Macedonia	N41 08.778 E20 59.467	d5 (5)	2-fragment
JX631146	*L. dalm.* ssp. *dalmatica*	Medun, Montenegro	N42 28.370 E19 21.708	d6 (2)	2-fragment
JX631147	*L. dalm.* ssp. *dalmatica*	Medun, Montenegro	N42 28.370 E19 21.708	d7 (1)	2-fragment
JX631148	*L. dalm.* ssp. *macedonica*	Florina, Greece	N40 45.310 E21 10.070	d8 (25)	2-fragment
JX631149	*L. dalm.* ssp. *macedonica*	Florina, Greece	N40 46.848 E21 22.531	d9 (2)	2-fragment
JX631150	*L. dalm.* ssp. *macedonica*	Florina, Greece	N40 47.005 E21 21.664	d10 (2)	2-fragment
JX631151	*L. dalm.* ssp. *macedonica*	Florina, Greece	N40 47.005 E21 21.664	d11 (1)	2-fragment
JX631152	*L. genist.* ssp. *genistifolia*	Limnotopos, Greece	N40 54.836 E22 38.926	d12 (1)	2-fragment
JN037642*	*L. genist.* ssp. *genistifolia*	Minićevo, East Serbia	N43 42.226 E22 18.134	g1 (1)	2-fragment
JN037643*	*L. genist.* ssp. *sofiana*	Harmanli, East Bulgaria	N41 56.438 E25 52.066	g2 (2)	2-fragment
JN037645*	*L. genist.* ssp. *genistifolia*	Slankamen, North Serbia	N45 08.343 E20 15.042	g3 (1)	2-fragment
JN037646*	*L. genist.* ssp. *genistifolia*	Preševo, South Serbia	N42 15.663 E21 42.100	g4 (1)	2-fragment
JN037647*	*L. genist.* ssp. *genistifolia*	Aleksinac, East Serbia	N43 34.480 E21 40.298	g5 (2)	2-fragment
JN037649*	*L. genist.* ssp. *genistifolia*	Niš, Central-East Serbia	N43 19.240 E21 59.410	g6 (6)	2-fragment
JN037653*	*L. genist.* ssp. *genistifolia*	Niš, Central-East Serbia	N43 19.240 E21 59.410	g7 (2)	2-fragment
JN037655*	*L. genist.* ssp. *genistifolia*	Kiskunság, Nat Park Hungary	N46 51.946 E19 24.003	g8 (6)	2-fragment
JN037661*	*L. genist.* ssp. *genistifolia*	Cegledbercel, Hungary	N47 13.204 E19 41.571	g9 (1)	2-fragment
JN037662*	*L. genist.* ssp. *genistifolia*	Cegledbercel, Hungary	N47 13.204 E19 41.571	g10 (1)	2-fragment
JX631153	*L. genist.* ssp. *genistifolia*	Primorsko, Bulgaria	N42 18.560 E27 37.396	g11 (1)	2-fragment
JX631154	*L. genist.* ssp. *genistifolia*	Harmanli, Bulgaria	N41 53.117 E25 52.373	g12 (1)	2-fragment
JX631155	*L. genist.* ssp. *genistifolia*	Jasnaja Poljana, Bulgaria	N42 16.501 E27 38.303	g13 (1)	2-fragment

* Sequences of mtCOII gene reported in previous study (Toševski et al. 2011)

[a] Haplotypes designations are according to Toševski et al. (2011). New haplotypes are designated with the next ascending number within group (v—*L. vulgaris* associated *M. janthinus*, s—*M. janthinus* 'speciosa' genotype, d—*L. dalmatica* associated *M. janthiniformis*, g—*L. genistifolia* associated *M. janthiniformis*, gd—*L. genistifolia/dalmatica* associated *M. janthiniformis*). Haplotype frequency based on current study and previous research (Toševski et al. 2011)

[b] Number of restriction fragments in the *Hpy*188III-*Mnl*I RFLP pattern

Results

Haplotype diversity

To determine haplotype diversity on mtCOII gene, altogether 137 newly collected specimens from central and southeastern Europe belonging to the *M. janthinus* species complex associated with *L. vulgaris*, *L. genistifolia/dalmatica* and specimens associated with *L. vulgaris* that belong to 'speciosa' genotype were analyzed and compared with formerly identified haplotypes (Table 2). In the previous study (Toševski et al. 2011), a total of 15 *M. janthinus* haplotypes associated with *L. vulgaris* (v1–v15), 19 *M. janthiniformis* haplotypes associated with *L. genistifolia/dalmatica* (g1–g10, d1–d5, gd1–gd4), and three haplotypes designated as 'speciosa' group genotype associated with *L. vulgaris* (s1–s3) were recorded. The analysis of additional material revealed the existence of another five haplotypes within *M. janthinus*, ten within *M. janthiniformis* (seven associated with *L. dalmatica* and three with *L. genistifolia*), but no new haplotypes within the 'speciosa' group genotype (Table 1). In total, 20 *M. janthinus s.s* haplotypes have been recorded out of 106 specimens from *L. vulgaris* sampled from a wide range of its central and southeastern European distribution, 29 *M. janthiniformis* haplotypes out of 198 specimens from its southeastern European distribution associated with *L. genistifolia* and *L. dalmatica* and three haplotypes belonging to the 'speciosa' genotype in ten sequenced specimens that originated from northern Switzerland, central Hungary and eastern Serbia (Table 2).

Sequence comparison and putative restriction maps

Sequence comparison of full length COII gene (678-bp) of the 52 haplotypes identified within the *M. janthinus* species complex revealed genetic variability represented with 59 variable, 27 singleton and 32 parsimony-informative sites. Out of these, only 11 sites could be used as informative for the discrimination of *M. janthinus* from *M. janthiniformis* and/or 'speciosa' genotypes or for the discrimination of *M. janthiniformis* from the 'speciosa' genotype (Table 3). All new haplotypes of *M. janthinus* and *M. janthiniformis* had a specific set of eight nucleotides defined as being species-specific in the original description (Toševski et al. 2011). These positions were used for the identification of restriction enzymes that could be used in the diagnostic assay.

In silico restriction analyses allowed us to identify *Hpy*188III and *Mnl*I as suitable enzymes for the practical and reliable procedure of molecular identification and differentiation of *M. janthinus s.s.* from *M. janthiniformis*. In addition, this procedure enables the discrimination of individuals belonging to the 'speciosa' genotype. Based on the putative restriction map of the COII gene sequences (Fig. 1), all *M. janthinus s.s.* and 'speciosa' specimens have two recognition sites for *Hpy*188III endonuclease (TC′nn_GA), at the 433- and 548-bp positions of the TL2-J-3038/TK-N3782 amplicons, while sequences of *M. janthiniformis* have only one recognition sequence for this enzyme at the 433-bp position. They lack the 548-bp restriction site due to a nucleotide substitution from A

Table 3 MtCOII gene variable sites informative for differentiation of *M. janthinus* species complex

Mecinus janthinus s.l.	Position of variable sites within the COII gene[a]										
	65	*171*[b] *(225)*	186	*202*	231	*252*	419	427	*489*[c] *(548)*	498	558
M. janthinus	*T*	*G*	G	*T*	C	*G*	A	A	*A*	A	T
M. janthiniformis	*A*	*A*	G	*C*	C	*A*	A	A	*G*	A	T
M. janthinus 'speciosa'	*A*	*A*	A	*T*	T	*A*	G	G	*A*	G	C

Nucleotide positions defined as being species-specific for identification of *M. janthinus* and *M. janthiniformis* (Toševski et al. 2011) are highlighted in italics

[a] Nucleotide position accounted from the beginning of the COII gene. Restriction sites within the TL2-J-3038/TK-N3782 PCR amplicon are given in parentheses

[b] Recognition site for proposed diagnostic enzyme *Mnl*I (CCTCNNNNNN_N′)

[c] Recognition site for proposed diagnostic enzyme *Hpy*188III (TC′NN_GA)

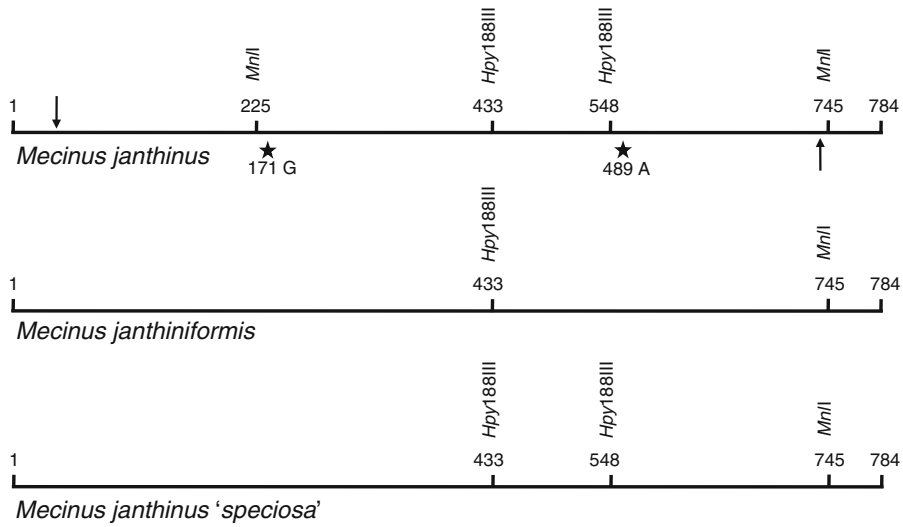

Fig. 1 Putative restriction map for *Mnl*I (CCTCNNNNNN_N′) and *Hpy*188III (TC′NN_GA) endonucleases based on TL2-J-3038/TK-N3782 delineated sequences of mtDNA fragments encompassing complete COII gene (indicated with vertical arrows) of *Mecinus janthinus s.s.*, *M. janthiniformis* and *M. janthinus s.l.* 'speciosa' genotype. Asterisks are indicating nucleotide positions of informative sites (accounting nucleotides from the beginning of the COII gene) enabling species-specific identification of *M. janthinus* and *M. janthiniformis* and differentiation from 'speciosa' genotype

to G at the 489-bp of the COII gene (Fig. 1; Table 3). This nucleotide position is interspecific informative and its substitution is species-specific for *M. janthiniformis* (Table 3). Consequently, this enzyme enables the discrimination of *M. janthinus* from *M. janthiniformis*, but not *M. janthinus* from 'speciosa' group genotypes. For the *Mnl*I restriction enzyme (CCTCnnnnnn_n′) all three groups of sequences have one recognition site at 745-bp position generating fragments of 745- and 39-bp in length. However, only *M. janthinus s.s.* has an additional *Mnl*I restriction site at the 225-bp position, which makes its restriction pattern unique. This additional *Mnl*I restriction site on the COII gene is a consequence of a nucleotide substitution from A to G at position 171, which distinguishes *M. janthinus* from *M. janthiniformis* (Toševski et al. 2011; Table 3).

RFLP diagnostic procedure

Based on *in silico* and in vitro restriction analysis of TL2-J-3038/TK-N3782 COII amplicons with *Hpy*188III and *Mnl*I endonucleases, we propose a diagnostic method using PCR-RFLP for the identification and separation of the *M. janthinus*, *M. janthiniformis* and 'speciosa' genotypes. Comparison of the sequence virtual analysis with actual digestion patterns of all 52 haplotypes confirmed the accuracy and reproducibility of the proposed methodology (Fig. 2a, b). All haplotypes had the expected restriction profiles which enabled their assignment to a species or genotype, solely based on the RFLP pattern. Single digestion with *Hpy*188III or *Mnl*I endonuclease was insufficient for the precise recognition of all three entities (Fig. 2). Restriction digestion with *Hpy*188III could separate only *M. janthinus* from *M. janthiniformis*, but not from the 'speciosa' genotype (Fig. 2). On the contrary, digestion with *Mnl*I enzyme enabled differentiation of *M. janthinus* from *M. janthiniformis*, but not *M. janthiniformis* from the 'speciosa' genotype. Therefore, only double digestion facilitates precise identification of each species or genotype.

In summary, double digestion of TL2-J-3038/TK-N3782 COII amplicons with *Hpy*188III and *Mnl*I enzymes generates a 4-fragment restriction profile in *M. janthinus s.s.*, a 2-fragment profile in *M. janthiniformis* and a 3-fragment profile in the 'speciosa' group of haplotypes (Fig. 2a). The fragment of 39-bp length is not included in the diagnostic RLFP patterns, since all restriction profiles possess this fragment and due to its length it is expected that it will be diffuse and hardly visible on actual RFLP gel (Fig. 2a, b). Thus, the RFLP pattern in specimens belonging to *M. janthinus s.s.* consists of four clearly separated and

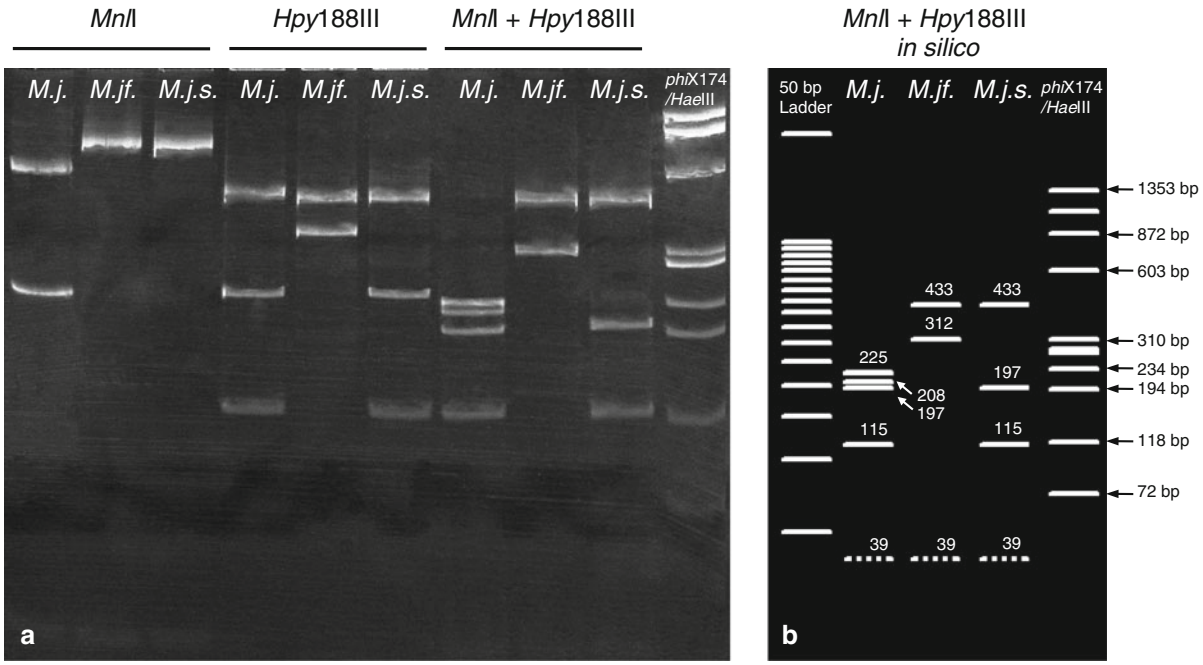

Fig. 2 Actual (**a**) and virtual (**b**) restriction profiles of the 784-bp COII gene amplified or *in silico* delineated by TL2-J-3038 and TK-N-3782 primer pair, single and double digested with *Mnl*I and *Hpy*188III restriction endonucleases. Abbreviations: *Mecinus janthinus s.s.* (*M.j.*), *Mecinus janthiniformis* (*M.jf.*), *Mecinus janthinus* 'speciosa' genotype (*M.j.s.*). Molecular weight markers: *phi*X174/*Hae*III digested (Fermentas) and 50 bp DNA Ladder (Invitrogen). Fragment sizes of the RFLP profiles and *phi*X174/*Hae*III marker are indicated on virtual gel image. Dotted lines in an *in silico* profile represent bands not visible or barely visible in actual experimental profile

visible fragments of 225, 208, 197 and 115 bp in length, *M. janthiniformis* two fragments of 433 and 312 bp and *M. janthinus* 'speciosa' genotype three fragments of 433, 197 and 115 bp (Fig. 2b).

Discussion

Methods developed for molecular identification of organisms have become important tools that can help to improve security and efficacy in using natural enemies in classical biological control of weeds (Gaskin et al. 2011). The presence of cryptic or sibling species within introduced and established populations of selected biological control agents are probably rare events, but it is an increasing point of concern. Since little effort has been dedicated to performing population genetic studies prior to the introduction of beneficial insect as biocontrol agents, the discovery of cryptic species within introduced arthropod agents has to be expected. Rauth et al. (2011) quoted only six studies that have characterized genetic variation within or among populations of weed biological control agents prior to their release. Moreover, little is done for the re-evaluation of the population genetics of introduced and already established agents. One of the rare examples of such a re-evaluation is a recently published study by Roehrdanz et al. (2011) on the evaluation of the genetic population status of several *Aphthona* species (Halticinae, Chrysomelidae) introduced in North America for the biological control of leafy spurge. The authors concluded that some populations of *Aphthona lacertosa*, one of the introduced species, are divergent enough to merit consideration of cryptic species and proposed further studies in order to determine physical traits, ecology and geographic origin of these specimens.

Historically, in nearly all the cases, the determination and taxonomic position of a potential biological control agent was based on traditional taxonomy and the typological species concept, whereby the identification was primarily based on morphological characters and on the authority of taxonomists specializing

in that particular taxa group. The same approach was applied in the selection of *M. janthinus* as a biological control agent against invasive toadflaxes, where identification of voucher specimens was confirmed by the taxonomist Dr L. Dieckmann, Eberswalde, Germany (Jeanneret and Schroeder 1991). The typological species concept and a general knowledge of the biology of *M. janthinus* that was available in literature during the time of pre-release studies has led to the introduction in North America of two different, host-associated weevil species from central and southeastern Europe (Toševski et al. 2011). *M. janthinus* and *M. janthiniformis* are morphologically distinguishable from each other by only a few very subtle characters, in contrast to strong genetic differentiation which was consistent also in specimens collected from sympatric populations of *L. vulgaris* and *L. genistifolia*. In addition, host plant associated genetic differentiations are supported by substantially higher offspring survival of the two *Mecinus* species on their respective host-plant species (Toševski et al. 2011). This example emphasizes the need for pre-release population genetic studies when selecting potential biological control agents.

The existence of 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 and a study is ongoing to determine the taxonomic status and European origin of invasive toadflaxes in North America. The redistribution and improvement of the biological control capacity of *Mecinus* weevils will be required to go through a proper selection of these cryptic species. Furthermore, genetic properties of the weevils should be complemented by the recognition of the genetic variability of the targeted toadflax populations. The proposed PCR-RFLP tool can avoid the use of inadequate weevil populations and minimize failure in redistribution and establishment of these beneficial weevils.

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