Revision of *Mecinus heydenii* species complex (Curculionidae): integrative taxonomy reveals multiple species exhibiting host specialisation

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Toševski I., Caldara R., Jović J., Baviera C., Hernández-Vera G., A. Gassmann & Emerson B.C. (2013). A combined taxonomic, morphological, molecular and biological study revealed that the species presently named *Mecinus heydenii* is actually composed of five different species: *M. heydenii* Wencker, 1866, *M. raffaeli* Baviera & Caldara sp. n., *M. laeviceps* Tournier, 1873, *M. peterharrisii* Toševski & Caldara sp. n., and *M. bulgaricus* Angelov, 1971. These species can be distinguished from each other by a few subtle characteristics, mainly in the shape of the rostrum and body of the penis, and the colour of the integument. The first four species live on different species of *Linaria* plants, respectively *L. vulgaris* (L.) P. Mill., *L. purpurea* (L.) P. Mill. *L. genistifolia* (L.) P. Mill. and *L. dalmatica* (L.) P. Mill., whereas the host plant of *M. bulgaricus* is still unknown. An analysis of mtCOII gene sequence data revealed high genetic divergence among these species, with uncorrected pairwise distances of 9% between *M. heydenii* and *M. raffaeli*, 11.5% between *M. laeviceps*, *M. heydenii* and *M. raffaeli*, while *M. laeviceps* and *M. peterharrisii* are approximately 6.3% divergent from each other. *Mecinus bulgaricus* exhibits even greater divergence from all these species, and is more closely related to *M. dorsalis* Aubé, 1850. Sampled populations of *M. laeviceps* form three geographical subspecies: *M. laeviceps* ssp. *laeviceps*, *M. laeviceps* ssp. *meridionalis* Toševski & Jović ssp. n. and *M. laeviceps* ssp. *corifoliae* Toševski & Jović ssp. n. These subspecies show clear genetic clustering with uncorrected mtDNA COII divergences of approximately 1.4% from each other.

Introduction

The species is a fundamental unit in biology and an important concept within any area of applied biology. Consequently, species delimitation underpins studies relating to biodiversity, conservation, evolution and ecology. Accurate species delimitation has become a key issue for food security and global trade due to tight administrative regulations that seek to restrict the presence of organisms that can endanger either state economies as agricultural pests, or regional biodiversity (Scheffer and Lewis, 2001; Brown, J.K. 2007, Krosch et al., 2012). Morphological taxonomy is deeply embedded within the taxonomic literature, largely due to a legacy whereby a typological species concept has

been followed in systematics for centuries. However, species determination is moving beyond single tools in taxonomy, such as the use of morphology alone, to integrative taxonomic approaches employing different character sets, such as genetic, ecological and behavioural data, to test hypotheses about species limits, where sampling focuses on representative coverage both within and among populations. One of the more demanding issues in taxonomy is the clarification of nomenclature questions, often complicated by the subjective statements of previous authors (Packer et al., 2009). Applying molecular tools, combined with the incorporation of morphological data, life history and ecological information provides us with a more data rich approach to resolve historical nomenclatural uncertainties. Unfortunately, although a growing number of studies are applying an integrative taxonomic approach, and incorporating important assumptions regarding species delimitation (Esterhuizen et al., 2013), especially between cryptic species, the nomenclatural issue, as a final act of clarification regarding systematic position of particular taxon or taxa group, frequently remains unresolved (Goldstein and DeSalle, 2010).

The characterization of species limits among populations of targeted biocontrol agents is *a priori* essential for the successful evaluation of their biological properties through host specificity testing, prior to the practical deployment of a biocontrol agent (Schaffner, 2001). In addition, classical biological control procedures are covered by strict standardized regulations that aim to minimize the accidental introduction of unwanted organisms. The existence of cryptic species, which are by definition difficult to distinguish or indistinguishable by phenotypic characteristics (Bickford, 2007), places extra demands in such applied studies. A biological control program for alien invasive toadflax species of European origin in North America, *Linaria dalmatica* (L.) Mill. (Dalmatian toadflax) and *L. vulgaris* (L.) Mill. (yellow toadflax) was initiated in 1987, and weevils from the genera *Mecinus* and *Rhinusa* (Mecinini, Curculionidae) were targeted as potential candidates for their biological control (Saner, 1991; Jeanneret, P., and D. Schroeder. 1992), with several weevil species subsequently introduced for this purpose (Wilson, 2005). Introduction of the stem-mining weevil *Mecinus janthinus* Germar was made in 1991 (De Clerck-Floate and Harris 2002), while *Rhinusa antirrhini* and *R. neta* that prefer Dalmatian toadflax were introduced by Canada in 1993, and *Rhinusa linariae* was approved for field release in the United States and Canada in 1995, for the biocontrol of both Dalmatian and yellow toadflax (Wilson, 2005).

Other weevils within the genus *Mecinus* Germar, 1821 associated with toadflaxes have also become of particular interest after the substantial impact of *M. janthinus* on Dalmatian toadflax (McClay and De Clerck-Floate, 2002). However, this is complicated by the taxonomic classification of

species within the tribe *Mecini* (Curculionidae, Curculioninae) which has proven to be difficult because of a lack of unambiguous morphological characters for species differentiation. Concerning *Mecinus*, the genus comprises only Palaearctic species and is presently composed of 47 species accordingly to a recent taxonomic revision by Caldara & Fogato (2013). All species live on Plantaginaceae, mostly associated with plants of the genus *Plantago*, while about one quarter of species live on plants of the genera *Linaria*, *Antirrhinum* and *Anarrhinum* (Caldara & Fogato 2013). In the middle of 2000th, another stem-boring weevil, *Mecinus heydenii* Wencker (1866), was investigated as a potential biological agent for control of invasive yellow toadflax populations in North America (Tosevski et al., 2004). Wencker (1866) described *M. heydenii* noticing that the taxon is characterized by a blue colour of the dorsal integument and a strongly curved rostrum. If these characters were considered together, they distinguish *M. heydenii* from all other previously described species of *Mecinus*. A few years later Tournier (1873) described *M. laeviceps* and reported that this species appeared to be very closely related to *M. heydenii*. Subsequently, two other taxa were described as closely related to the previous two: *M. venturensis* Hoffmann, 1940 and *M. bulgaricus* Angelov, 1971. Several authors (Hoffmann, 1958; Smreczyński, 1960; Alziar, 1975; Smreczyński, 1976) discussed the taxonomic validity of these taxa with different conclusions. Finally, Arzanov (2000) considered all of these taxa to belong to a unique "variable" species, *M. heydenii*. However, host specificity and population genetic analyses strongly suggest the existence of cryptic species within *M. heydenii* populations associated with diverse toadflax species (Toševski et al., 2007). This observation of potential cryptic species within *M. heydenii* must be taken into account, if it is to be considered as a biological control agent, especially after recent published studies demonstrating host associated cryptic species within *M. janthinus* (Toševski et al. 2011) and another member of the Mecini, *Rhinusa antirrhini* (Hernandez-Vera et al., 2010). Therefore, in order to clarify taxonomical and nomenclatural discrepancies and to broaden knowledge of their biology, we use morphological, molecular and bionomical approaches to evaluate species limits among *M. heydenii* populations associated with different toadflaxes in Europe.

Material and Methods

Specimens examined

This study was based on the examination of type specimens of *M. heydenii* (DEI) and *M. laeviceps* (MHNG), specimens from private and public collections, and newly collected material. Several

hundred specimens were examined in order to estimate the geographical distributions of the studied taxa. Specimens of *M. heydenii* s.l. for molecular analysis were reared or collected from their host plants during several surveys performed from west Europe to east Turkey between 2008-2012 (Table 1). The weevils used for DNA analysis were stored just after being collected in 96% ethanol at 5-7° C until DNA extraction. After DNA extraction weevils were prepared as dry voucher specimens for taxonomic study. The collections housing material studied in this revision are abbreviated as follows (with their curators in parentheses):

BMNH - The British Museum of Natural History (Maxwell Barclay)

CABIC - CABI Europe collection, Delémont, Switzerland.

DEI Deutsches Entomologisches Institut, Müncheberg, Germany (Lutz Behne)

ITC - Ivo Toševski collection, Novi Beograd, Serbia

MHNG - Muséum d'Histoire Naturelle de Genève, Switzerland (Ivan Löbl)

MNHN - Muséum National d'Histoire Naturelle, Paris, France (Hélène Perrin)

MZHF - University of Helsinki, Zoological Museum, Helsinki, Finland (Jyrki Muona, Hans Silfverberg)

RCC - Roberto Caldara collection, Milano, Italy.

ZIN - Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia (Boris A. Korotyaev)

Morphological study

We considered the following characters as possibly diagnostic according to Caldara & Fogato (2013): body length (rostrum excluded), shape of head, eye, antennae, rostrum, prothorax, elytra and abdomen; colour, sculpture and vestiture of rostrum; pronotum and elytra; shape of penis and spermatheca. In the following treatment of the species, due to the great similarity among them, we prefer to report an accurate diagnosis instead of a complete description, in order to avoid those characters common to all the species of the genus and of the studied group.

Host suitability study

Host specificity studies were conducted with weevils associated with *L. vulgaris* (= *M. heydenii* s.s.) and *L. genistifolia*, because these weevil populations could be collected at several sites in sympatry (Table 1). *M. heydenii* s.s. specimens associated with *L. vulgaris* were collected near the

village of Mihajlovac (N44 20.054 E22 28.387, 153 m). Well sclerotized adult specimens were extracted by stem dissection from their respective host plant at the beginning of August 2008. Individual adults were placed inside separate cages (34 x 40 x 25 cm) containing naturally growing *L. vulgaris* and allowed to overwinter in an outdoor insectarium. No-choice oviposition and larval development tests were conducted from 22 March 2009 onwards, and control and test plants were exposed to single mated females until the beginning of June. Weevils from *L. genistifolia* was collected near Slankamen (N45 08.521 E20 14.745, 199 m) in early May 2009 and no-choice oviposition and larval development tests with these weevils were conducted until the beginning of June.

A total of 11 mated females from *L. vulgaris* and 11 mated females from *L. genistifolia* were used in this study, of which three females of both weevil populations were used as a no food control to determine maximum starvation period. Both weevil populations were tested on *L. vulgaris* and *L. genistifolia*, where authentic field host plants were used as a control. During testing, females were transferred to fresh plants approximately every 4-7 days for oviposition. Female longevity (in days) was recorded until mid-June. All plants were dissected for larval, pupal and adult development during first week of August. Larval development was assessed by total and mean development rate on control and test plants, considering tested females as replicates.

Polymerase chain reaction (PCR) amplification and sequencing

DNA extraction. Individual weevils were punctured between the 2nd and 3rd thoracic sternites and total DNA was extracted using the QIAGEN Dneasy® Blood & Tissue Kit, according to the manufacturers instructions. The mitochondrial 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'-GAGACCATTACTTGCTTTTCAGTCATCT) (EVA-Harrison Laboratory, Cornell University, Ithaca, NY, USA). Polymerase chain reactions (PCR) contained NH₄ buffer (1x), 2.5 - 3.5 mM MgCl₂, 0.2mM of each dNTP, 0.1 - 0.5 µM of each primer and 0.5 U of Taq polymerase (Fermentas) in a 25µL 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. Amplified products were sequenced on an ABI Prism 3700 automated sequencer using the commercial services of BMR Genomics (University of Padova, Italy) and Macrogen (Seoul, Korea).

Evolutionary tree construction, haplotype network construction and estimation of divergence times

Phylogenetic analyses. Sequences were edited with FinchTV v.1.4.0 (www.geospiza.com), and aligned with ClustalW within the Mega4 software (Tamura et al., 2007), and aligned sequences were trimmed to 678 bp in length. Maximum parsimony and neighbour-joining trees were generated in PAUP* version 4.0b10 (Swofford 2002) using the evolutionary model of nucleotide substitution that best fits the data, determined with jMODELTEST version 2.1.1 (Darriba et al, 2012), using the default values. Five hundred bootstrap replicates were performed to assess branch support within the resulting tree topologies of both analyses. Bayesian analyses were carried out with the program MRBAYES version 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). We conducted two simultaneous runs for 300 000 generations, sampling every 100 generations, with a heating parameter value of 0.20 and a ‘burn-in’ of 25%, using the model of nucleotide substitution determined by jMODELTEST, with all other priors set to default values.

Haplotype network construction. 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 for *M. heydenii* s.l. populations in association with different *Linaria* host plants using statistical parsimony (Templeton *et al.*, 1992) with a confidence limit of 95%.

Estimation of divergence times. In the absence of geological and/or fossil calibration points related with weevils from *M. heydenii* species complex, we have taken a Bayesian approach using a generalized clock to estimate the age of the most recent common ancestor (mrca). According to literature data, the mitochondrial rates have been proposed for arthropods in the range of 1.2-4.9% pairwise divergence per million years (Desalle et al. 1987; Brower 1994; Caccone & Sbordoni 2001; Wares 2001). We apply a mean rate estimate of 3.05% pairwise divergence per million years (Ma) previously used for similar analyses within the *Rhinusa antirrhini* (Paykull, 1800) complex, a related taxon from the tribe Mecinini (Hernandez-Vera et al, 2010).

In order to estimate the divergence time of the most recent common ancestor (mrca) of the *M. heydenii* complex, we used BEAST version 1.7.4 (Drummond et al., 2012). For our analyses we

used an uncorrelated lognormal relaxed molecular-clock model in BEAST with the average number of substitutions per site across the tree averaged to be 1.525 per million years, but with rates for individual branches unconstrained. Substitution model parameter values were HKY+I, selected according to the results of jMODELTEST, with unconstrained prior distributions. A demographic model of constant population size was applied, in the form of a coalescent prior, to clades conforming to genetic variation within host associated lineages, and a Yule tree prior was used for the basal branches connecting these. Input files were generated with BEAUTI version 1.7.4 (Drummond et al., 2012). Two runs, consisting of 20 000 000 generations each and sampling every 1000 generations, were performed and combined, checking sampling, mixing and convergence to a stationary distribution. Estimated times to the most recent common ancestor (mrca) for host-associated mitochondrial lineages was assessed from both runs using Tracer 1.5.0 (<http://beast.bio.ed.ac.uk>) as mean values in million years (Ma) with 95% highest and lowest posterior density intervals (HPD).

Results

Surveys and collections

Surveys between 2008-2011 allowed us to collect and study *de novo* sampled weevil populations from 6 different *Linaria* species or populations: *L. vulgaris*, *L. genistifolia*, *L. dalmatica* ssp. *dalmatica*, *L. dalmatica* ssp. *macedonica*, *L. corifolia*, *L. purpurea* and *L. simplex*. Sampled material that was used for genetic analysis is presented in Table S1 (supporting information), while the geographic locations of sampling sites are reported in Fig. 1. Adults of *M. heydenii* s.s. were reared or collected from *L. vulgaris* from five sites in northern and eastern Serbia, two sites in each of southern France, western Germany, central Hungary, and north-eastern Romania, and one site in the Ukraine. Adults from *L. genistifolia* were reared or collected from two sites in central Hungary, one in the Czech Republic, two in northern Serbia, three in eastern Serbia and one site in eastern Bulgaria (Appendix S1). Within three sites (central Hungary, northern Serbia and eastern Serbia) weevils were sampled from both *L. vulgaris* and *L. genistifolia* growing in sympatry. Adult weevils were reared from *L. dalmatica* ssp. *macedonica* from 6 sites on the Pelagonian plateau in Macedonia and several geographically proximate sites in northern Greece. *L. dalmatica* ssp. *dalmatica* was sampled from a single coastal site in Montenegro. Weevils were sampled from *Linaria purpurea* from two sites on Mt. Etna (Sicily, Italy). Weevils were sampled from *Linaria corifolia* Desf., at one site in north-eastern Anatolia, Turkey. One adult determined as *M. dorsalis*

was collected in southern France (Eygalières) as well as two L2 larvae of this species were extracted from solid galls induced at shoot-base on *L. simplex*. In addition, we have collected single specimen of *M. bulgaricus* near the town Harmanli (N41 53.117 E25 52.373, 310 m) which is the type locality of this species according to Angelov (1971).

Host specificity study

No females lived longer than five days in the no plant control. Offspring survival rates were similar across both host-plant groups when individuals were reared on the host-plant species they were sampled from, i.e. 180 adults on *L. vulgaris* and 96 on *L. genistifolia*, respectively. No larval development or only negligible survival rate was registered when individuals were raised on the alternative host (Table 1). In addition, *L. genistifolia* plants presented to females originating from *L. vulgaris* (= *M. heydenii* s.s.) for oviposition resulted in 121 strong hypersensitive stem reactions that precluded any possibility for larval development.

Mitochondrial COII analyses

The length of complete COII gene was 684 bp, with the exception of specimens collected and reared from *L. purpurea*, where the complete COII gene was 681 bp. The final alignment of the COII sequences consisted of 678 bp, with a total of 177 (26.1%) polymorphic nucleotides of which 126 are parsimony informative. The maximum ingroup genetic distance was 14.6% (uncorrected) and all three phylogenetic analyses revealed substantial genetic divergence associated with host plant use (Table 2).

Across 107 sequenced specimens, a total of 18 different haplotypes were identified within the *M. heydenii* complex, collected from 6 different toadflax species and populations, with an average pairwise divergence across all sequences of 8.7% (Table 2). Sequences are available from GenBank under accession numbers xxxxxxxx-xxxxxxx (Table S1). The Akaike information criterion revealed the Hasegawa, Kishino and Yano model with invariant sites (HKY+I) to be the best model

to describe the pattern of nucleotide variation, and this was employed for the estimation of pairwise genetic distances for a neighbour-joining tree, and for Bayesian analysis.

Mecinus heydenii s.s. populations reared and collected from *L. vulgaris*, revealed a total of 8 haplotypes (hey1-hey8) with a mean pairwise divergence of 0.4% (Table 2). Less genetic diversity (mean 0.2%) was recorded among specimens collected from *L. genistifolia* from four sites north of the rivers Danube and Sava (laev1-laev3), and no haplotype diversity was recorded among individuals sampled from *L. genistifolia* south of these rivers, *L. corifolia* in north-eastern Turkey and *L. purpurea* in Sicily island. Only two haplotypes, with a divergence of 0.3%, were sampled from both *L. dalmatica* ssp. *macedoniaca* in southern Macedonia-northern Greece and *L. dalmatica* ssp. *dalmatica* in Montenegro.

All three phylogenetic analyses resulted in an identical tree topology (Fig 2). Four mitochondrial lineages were clearly defined within *M. heydenii* s.l. populations (Fig 2, node III) with the monophyly of each supported by bootstrap values in excess of 90%. Two out of three closely related lineages, with genetic divergence below 1.5%, were associated with *L. genistifolia* (populations north and south from rivers Sava and Danube), while third lineage was associated with *L. corifolia* from north-east Turkey (Fig 2, nodes a and b). Two species, one of which is *M. dorsalis* sampled from *L. simplex*, and the second identified as *M. bulgaricus* (host plant unknown) formed a sister group to the *M. heydenii* complex, hereby designated as the *M. dorsalis* species subgroup, subordinate to *Mecinus heydenii* species complex.

The haplotype network constructed using statistical parsimony revealed no connections at confident limit of 95% between mitochondrial lineages associated with *L. vulgaris*, *L. purpurea*, *L. dalmatica*, *L. simplex* and *L. genistifolia* - *L. corifolia*. A total of 8 haplotypes from *L. vulgaris* revealed no ambiguous connections inside their network, also in lineages associated with *L. genistifolia* and *L. corifolia* (Fig. 3).

Estimation of divergence times

Using *Rhinusa herbarum* (Brisout, 1862) as an outgroup we assessed divergence times among populations sampled within the *M. heydenii* species complex. High values were achieved for effective sample sizes for all parameters within our BEAST analysis, and age estimates for nodes of importance are presented in Table 3. The *M. heydenii* and *M. dorsalis* species groups (node II) are

estimated to have diverged from each other approximately 8.1 Ma (95%HPD: 5.1-10.8). Divergence within these two groups (nodes III and IV) is estimated to have initiated approximately 5.4 Ma (95% HPD:3.5-7.66) and 4.8 Ma (95% HPD: 2.1-7.5), respectively. The divergence between mtDNA lineages associated with *L. vulgaris* (= *M. heydenii* s.s.) and *L. purpurea* (node V) is estimated to have occurred approximately 3.4 Ma (95% HPD: 1.7-4.9), while mtDNA lineages associated with *L. dalmatica*, *L. genistifolia* and *L. corifolia* (node VI) are estimated to have diverged approximately 2.8 Ma (95% HPD: 1.5-4.1). The latter clade exhibits substantial divergence in regard to northern populations associated with *L. genistifolia* as host plant (node a) and southern populations associated with *L. genistifolia* and *L. corifolia* (node b), with the ages of each node estimated to be 0.8 Ma (95% HPD: 0.3-1.4) and 0.5 Ma (95% HPD: 0.2-0.9), respectively (Table 3).

Morphological study

In contrast to substantial mtDNA lineage differentiation, indicative of host associated cryptic speciation events, the specimens collected from different host *Linaria* species are morphologically very similar, making it very difficult to differentiate them only on the basis of phenotypic characteristics. The shape of the rostrum (Fig 4), morphology of the penis (Fig 5), average body length and subtle variation in the blue sheen of the pronotum and elytra, are subtle morphological characters that usually the separation of specimens associated with *L. vulgaris*, *L. genistifolia*, *L. dalmatica* and *L. purpurea*. *Mecinus dorsalis* and *M. bulgaricus* are well distinguished from members of *M. heydenii* species group, possessing black integument and lacking a metallic blue sheen. However, both species are morphologically themselves very similar to each other.

Treatment of the species

According to Caldara & Fogato (2013), *M. heydenii* s.l. and *M. dorsalis*, together with *M. aubei* Desbrochers des Loges, 1893 and *M. tavaresi* Hoffmann, 1958 (the latter two species not treated in the present study), all of them with a distinctly elongate body and black or blue dorsal integument, form a monophyletic group based on several apomorphies: rostrum in basal half strongly and abruptly curved, outer tarsal claw in male reduced or almost indistinct, body of penis with moderately to distinctly sharp apex, spermatheca with insertion of ductus placed perpendicular to length of nodus. Results obtained from combined morphological, molecular and biological data

allow us to recognize the existence of two species subgroups within the *M. heydenii* species complex, with six morphologically close related taxa.

***Mecinus heydenii* species subgroup**

***Mecinus heydenii* Wencker**

Mecinus heydenii Wencker, 1866: 130. Tournier, 1874: 41. Bedel, 1885: 148. Desbrochers des Loges, 1893: 59. Reitter, 1907: 8; 1916: 225. Hustache, 1931: 401, 405. Van Emden, 1938: 21. Hoffmann, 1958: 1267, 1269. Péricart, 1974: 65. Alziar, 1975: 5. Smreczyński, 1976: 4. Lohse & Tischler, 1983: 262. Caldara et al., 2010: 241. Caldara & Fogato, 2013 in press. Caldara et al., 2013 in press.

Type series. *Mecinus heydenii* was described from specimens collected by Heyden at Frankfurt (Germany) and by Wencker at Haguenau (Alsace, eastern France). In accordance with Caldara et al. (2010) here use the original spelling instead of the spelling *heydeni*, erroneously used by many authors. We examined the lectotype (DEI) and two paralectotypes (DEI and MNHN) of *M. heydenii* (des. Caldara & Fogato, 2013), all collected at Frankfurt.

Synonyms. *Mecinus heydenii venturensis* was described from specimens collected at Malaucène and Saint-Colombe at the foot of Mont Ventoux (Provence, southern France), of which we examined the holotype (MNHN). We here confirm the opinion by Hoffmann (1958), Smreczyński (1976) and Caldara & Fogato (2013), who considered this taxon as a synonym of *M. heydenii*.

Non-type specimens. We can confirm that all the specimens from France, Belgium, Denmark, Germany, and Northern Italy quoted in detail by Caldara & Fogato (2013) really belong to this species. Moreover we examined the following additional specimens: **Germany:** Kreimbach, on *L. vulgaris*, 25.04.2010, leg. Toševski (4 ITC); Odenbach, on *L. vulgaris*, 25.04.2010, leg. Toševski (1, ITC). **Czech Republic:** Bohemia, Most, Lišnice 30.VI.1985, leg. Schön (1, RBC). Slovakia: Hegy Farok, 13.VI.1984, leg. Borovec (1, RBC). **Hungary:** Budaörs, 9.V.1980, leg. Tyrner (1, RBC); Simontornya, on *L. vulgaris*, 2.05.2010, leg. Toševski (4 ITC). **Serbia:** Dobanovci, Srem district, ex l., ex *L. vulgaris*, 11.06.2006, leg. Toševski (1 ITC); Nova Pazova, Srem district, ex l., ex *L. vulgaris*, 11.06.2008, leg. Toševski (1, ITC); Mihajlovac, Negotin, East Serbia, ex l., ex *L. vulgaris* 20.07.2008, leg. Toševski (50, ITC, 50 RCC); Zaječar, East Serbia, ex l., ex *L. vulgaris*

15.07.2007, leg. Toševski (50, ITC, 50 RCC); Suvodol, Knjaževac, East Serbia, ex l., ex *L. vulgaris*
20.07.2007, leg. Toševski (20, ITC, 20 RCC). **Romania:** Moimești, Popricani, Iasi, on *L. vulgaris*,
30.04.2010, leg. Toševski (20, ITC, 15 RCC); Valea Lupului, Iasi, on *L. vulgaris*, 30.04.2010, leg.
Toševski (1, ITC). **Bulgaria:** Rodopi or., Peruštica, 19.V.1985, leg. Kadlec & Voříšek (1, OVC).
Ukraine: Kiev, on *L. vulgaris*, 19.05.2012, leg. Gassmann (2, ITC).

Diagnosis Body length 1.6-2.8 mm [males: 2.4 ± 0.2 (1.9-2.7) n=24; females 2.6 ± 0.1 (2.3-2.8) n=24]. Scales of dorsal vestiture whitish and well visible along middle and sides of pronotum and on elytral interstriae. Rostrum in lateral view very arcuate, strongly curved at antennal insertion, more curved and moderately longer in female (Figs. 4 –A, –B). Second segment of antennal funicle in female 1.5X as long as wide. Pronotum black with bronze reflections. Elytra 1.70-1.75X longer than wide, 2.50-2.60X longer than pronotum. Penis with sides slightly narrowed from base to apex, ending in a distinctly constricted, moderately long, subacute point (Fig. 5 –A).

Biology. The host plant is *Linaria vulgaris*. *Mecinus heydenii* is univoltine and overwinters in the adult stage. Intense adult feeding on the host plant starts in early March. Copulation occurs shortly after. The egg-laying period lasts from mid-March until mid-June. Oviposition occurs on actively growing young shoots usually in the top or middle stem part but rarely in the stem base. Females lay 1-6 eggs at once, distributed along the shoot. Before ovipositing, the female prepares a shallow hole where the egg will be deposited. During oviposition, the female secretes a fluid that provokes a primitive shoot swelling characterized by a narrow zone of stem hypertrophy leading to a semi-gall formation. The larvae will continuously feed on this gall-like tissue, so they usually do not leave the larval chamber to mine the shoot. Larval feeding often entirely stops shoot growth resulting in stunted shoots. Later, larval development induces minor and weakly visible gall-like alternations on the stem. Before pupation, larvae prepare an exit hole leaving a thin layer of stem epidermis at the end of the exit hole. After eclosion, the adults continue feeding on the gall-like tissue inside the larval chamber. Because the oviposition period extends over three months, different stages are often observed with the shoots. The adults do not leave the larval chamber before early August, during which all adults will simultaneously leave their host plant over a two week period. According to observations performed with caged adults (unpublished data), intensive adult feeding occurs until late autumn. In addition to their small size, the adults of *M. heydenii* exhibit extreme cryptic behavior, which makes them difficult to collect.

Distribution. The presence of this species was confirmed for the following countries: France, Belgium, Germany, Poland, Denmark, Sweden, Czech Republic, Slovakia, Hungary, Italy, Serbia, Bulgaria, Romania, Ukraine.

Remarks and comparative notes. This species is distinguishable from all the other species of the *M. heydenii* species complex by the rostrum strongly curved from base to apex in both sexes and the penis with slightly narrowing sides. It is the unique species of the group present in northern Europe, from Germany to Sweden.

***Mecinus laeviceps* ssp. *laeviceps* Tournier**

Mecinus laeviceps Tournier, 1873: 85; 1874: 42. Reitter, 1907: 8. Hoffmann, 1958: 1267, 1270. Smreczyński, 1960: 76; 1976: 4. Lohse & Tischler, 1983: 262. Arzanov, 2000: 1087. Caldara & Fogato, 2013 in press.

Type series. *Mecinus laeviceps* was described from specimens collected at Sarepta (southern Russia). Tournier (1873) reported that this species appears intermediate between *M. janthinus* (Germar, 1821) and *M. heydenii*, from which it differs by smooth head, pronotum with darker colour of the integument and finer and more separated punctures. Subsequently Hoffmann (1958) and Lohse & Tischler (1983) followed Tournier's opinion, whereas Smreczyński (1976), although tentatively, and Arzanov (2000) placed *M. laeviceps* in synonymy with *M. heydenii*. Arzanov (2000) reported to have also examined a male specimen from Sarepta (ZIN) which he inferred to belong to the type series, and which he designated as a lectotype. We examined this specimen and also another female specimen labelled “Sarepta / Collection E. Poncy” found at MHNG where part of Tournier's collection is deposited. Since this latter specimen also fits exactly with the original description, it is here considered as a paralectotype.

Non-type specimens. **Czech Republic:** Znojmo-Hradiště, 1.10.2009, leg. Stejskal (7, ITC); **Hungary:** Ceglédbercel, on *L. genistifolia*, leg. Toševski (3, ITC). **Serbia:** South Banat, Devojački Bunar, ex *L. genistifolia*, 29.09. 2007, leg. Toševski (3, ITC); Šušara, South Banat, Deliblatski pesak, ex *L. genistifolia*, leg. Toševski (30, ITC; 30 RCC); Slankamen, Srem, ex *L. genistifolia*, 20.08. 2008, leg. Toševski (20, ITC; 20, RCC).

Diagnosis Body length 2.4-3.3 mm [males: 2.8 ± 0.2 (2.4-3.3) $n=24$, females 2.9 ± 0.1 (2.8-3.2) $n=24$]. Scales of dorsal vestiture whitish and very visible along the middle and sides of the

pronotum and on the elytral interstriae. Rostrum in lateral view moderately and regularly curved from base to apex, moderately longer in female (Figs. 4, –C, –D). Second segment of antennal funicle in female 2.0X as long as wide. Pronotum black with bluish reflections. Elytra 1.70-1.75X as long as wide, 2.7-2.8X longer than pronotum. Penis with sides distinctly narrowed from base to apex, gradually constricted at apex ending with a shorter subacute point (Fig. 5, –B).

Biology. A univoltine species which develops in the stems of *L. genistifolia*. There is no other host plant known to date. In Serbia, the adults, which appear in the field during the second half of March, aggregate on young growing shoots causing significant damage to the shoot points and apical leaves. The weevils copulate from the end of March until mid-April. First oviposition occurs in mid April and is completed by the end of May. Usually females oviposit batches of 3 to 6 eggs into the lower to middle part of young growing shoots. Average fecundity of *M. laeviceps* is estimated to be 40-50 eggs per female. Infestation by larvae of *M. laeviceps* results in stunted growth of the whole shoot of *L. genistifolia*. There are three larval instars. First and 2nd instar larvae develop in the central part of the stem in a relatively short tunnel. Atypical tissue proliferation of parenchyma cells can be observed surrounding the feeding larvae as a consequence of cecidogenic secretion occurring during oviposition. On *L. genistifolia*, the stem surface becomes rugose, especially in heavily attacked shoots. Before pupation, larvae prepare an exit hole leaving a thin layer of stem epidermis. Pupation takes place in the somewhat expanded end of the short larval tunnel, just below the exit hole. The pupal stage lasts about 10-15 days. Newly emerged adults remain in the pupal chambers. During summer, adults feed within the pupal chambers. By the end of August, all adults leave their pupal chambers. Adults overwinter in the soil close to the host plant.

Distribution: Czech Republic, Hungary, northern Serbia, southern Russia.

Remarks and comparative notes. This species can be distinguished from *M. heydenii* by the rostrum in lateral view, which is less arcuate and more regularly curved in both sexes, and by the penis, which is more distinctly narrowed in the apical half and with a shorter point. It is more difficult to separate *M. laeviceps* s.l. from *M. raffaeli* and *M. peterharrisi* (see below), in which however the rostrum of the female is nearly straight in the apical portion. Moreover in *M. peterharrisi* the penis has a longer point and the body is often also longer. However it is noteworthy that whereas *M. laeviceps* s.l. is sympatric at least in part of its area of distribution with *M. heydeni* (Fig. 1), no sympatric distribution was recorded with *M. peterharrisi* and *M. raffaeli*.

We have studied a total of nine populations of *M. laeviceps laeviceps*: eight distributed in central and south-eastern Europe and one from eastern Turkey. Five populations that occur north of the Sava and Danube rivers (north Serbia, Hungary and south Czechia) are here referred to as the nominal subspecies of *M. laeviceps*. A total of three haplotypes were recorded within all “northern” populations suggesting a low level of genetic diversity (Fig 3, –C). The haplotype designated as *laev1* was widely distributed, present in all five populations north of the Sava and Danube rivers.

***Mecinus laeviceps* ssp. *meridionalis* Toševski & Jović ssp. n.**

Type series. Holotype, male: Serbia, Kladovo, Petrovo Selo, 10.07.2007, N44 37.623 E22 31.667, 158 m., ex *L. genistifolia*, leg. Toševski, DNA 2338, voucher SR2338, haplotype meri01, (deposited in BMNH). Paratypes and depositories are listed in supporting information S2). Paratypes: Serbia, Kladovo, Petrovo Selo, 10.08.2007, N44 37.623 E22 31.667, 158 m., ex *L. genistifolia*, leg. Toševski (5 ITC); Negotin, 10.07.2007, ex *L. genistifolia*, leg. Toševski (9 ITC); Negotin, 15.08.2009, leg. Toševski (15 ITC, 15 RCC, 5 BMNH, 5 MNHM); Bulgaria, Harmanli, 12.04.2012, on *L. genistifolia*, leg. Toševski (3 ITC, 3RCC, 1 BMNH).

Diagnosis Similar to *M. laeviceps* ssp. *laeviceps* except: body somewhat smaller, body length 2.3–3.0 mm [males: 2.7 ± 0.2 (2.3–2.9) $n=24$, females 2.7 ± 0.2 (2.4–3.0) $n=24$]; pronotum with less bluish reflection, sometimes bluish black or even with discrete bronze reflection.

Biology. There are no differences in biology between nominal subspecies and subsp. *meridionalis*. The host plant is for both subspecies *L. genistifolia*, a very common plant species on ruderal and arid sites in south-eastern Europe. In contrast to the more common nature of its host plant, populations of ssp. *meridionalis* are rare, local and disjunct. Behaviour of the adults and larvae, including larval development corresponds to those of the nominal subspecies.

Distribution. Serbia and Bulgaria (south of rivers Sava and Danube).

Remarks and comparative notes. There are no constant morphological differences between *M. laeviceps* ssp. *meridionalis* and *M. laeviceps* ssp. *laeviceps*, with the exception that the newly described subspecies is slightly smaller and possess less coloured integument. We have examined a total of four populations of ssp. *meridionalis*: three populations from East Serbia and one from

Harmanli (south-eastern Bulgaria). In all sequenced specimens from these “southern” populations (Fig. 3, –D), we have registered constant genetic divergence ($\approx 1.4\%$) from specimens belonging to the nominal subspecies. Both subspecies are sharply separated by the rivers Sava and Danube (Table 2, Fig. 3, –C). Constant and stabile genetic divergence strongly suggests absence of gene-flow between populations distributed north and south from these rivers. The stabile and massive geographic barrier over time, at first place river Danube, probably contributed to habitat fragmentation and separation of *M. laeviceps* populations in south-eastern Europe delimiting genetically distinguishable populations which represent an example of genetic diversification following allopatric pattern. According to uncorrelated lognormal relaxed molecular-clock model, nominal ssp. *laeviceps* and ssp. *meridionalis* have diverged approximately 0.78 Ma (Table 3, node a).

Etymology. The Latin adjective *meridionalis*, meaning “southern”, emphasizes the geographic distribution of this new subspecies south of the rivers Sava and Danube.

***Mecinus laeviceps* ssp. *corifoliae* Toševski & Jović ssp. n.**

Type series: Holotype, male: Turkey, Tortum, Deveyatağı, Erzurum prov., N40 23.630 E41 29.871, 1333 m, 12.07.2011, ex *L. corifoliae*, leg. Toševski, DNA IT-1410, voucher TR1410, haplotype cori01, (deposited in BMNH). Paratypes: 3 males, with the same labells as the holotype. .

Diagnosis Similar to *M. laeviceps* ssp. *laeviceps* except: body somewhat smaller, body length 1.9-2.1 mm [males n= 3]. Rostrum in male in lateral view more curved at middle instead of basal third. Female unknown.

Biology. The host plant of *M. laeviceps* ssp. *corifoliae* is *Linaria corifolia*, a glaucous, shrubby perennial plant with numerous decumbent or erect fertile stems 10-40 cm height. The females lay eggs (1-3) at the stem base of the young growing shoots, early in the spring. There are three larval instars. Larval development takes place in the central part of the stem pith, mostly in the basal part of the shoot. The presence of larvae is barely distinguishable, even though stems of this toadflax species are very fine, about 1 mm in diameter at the stem base. The pupa/adult chamber in the stem is also centrally situated, while sub-epidermal exist hole was not observed on stem surface.

Distribution: The host plant *Linaria corifolia* is a widespread and fairly common toadflax species in Anatolia (Turkey), most often present on calcareous screes or rocky slopes of the hilly mountains. We have examined over 30 *L. corifolia* populations from eastern Anatolia (Korkuteli district, Anthalya prov.) to the Kars and Ardahan provinces in the eastern part of Anatolian plateau. Even though an intensive survey was performed, only single population of weevils was recorded near the town of Tortum. This strongly suggests that populations of the ssp. *corifoliae* are rare and local, similar to observations for other *M. laeviceps* populations.

Remarks and comparative notes. We have never examined specimens of the other two subspecies as small as those of *M. laeviceps* ssp. *corifoliae*. However this apparently diagnostic difference must be confirmed by the examination of more numerous specimens of this last subspecies. It should be pointed out that one paratype (TR1413) lacks elytra, although the abdomen is well preserved, and another paratype (TR1412) lacks head and pronotum, but however the elytra are as long as those of the other specimens of the type series. It will be also important to find female specimens in order to confirm the little differences in the shape of the rostrum shown between males of this subspecies and those of the other two subspecies.

Two substantial differences define the newly described ssp. *corifoliae* from ssp. *laeviceps* and ssp. *meridionalis*. Firstly, different host plant, and secondly, the constant genetic divergence on mtCOII gene of 1.3% and 1.5% in regard to ssp. *laeviceps* and ssp. *meridionalis*, respectively. Although host association with another toadflax species and significant genetic divergence suggest that specimens associated with *L. corifolia* may represent a well differentiated taxon, the estimated divergence time between ssp. *laeviceps* and ssp. *corifolia* is 0.51 Ma (Table 3, node b). However, estimated divergence time is lower than between ssp. *laeviceps* and ssp. *meridionalis* although their populations share same host plant. Thus, it is more justifiable to treat ssp. *corifolia* as a taxonomic entity subordinate to *M. laeviceps*. However, we don't exclude that further study with weevil populations associated with *L. corifolia* may lead to different conclusion.

Etymology. The new subspecies is named after *Linaria corifolia*, in order to emphasize its host associations with this toadflax.

***M. peterharrisi* Toševski & Caldara sp. n.**

Type series. Holotype, male, Macedonia, Pretor, 7.08.2007, N41 03.780 E21 03.118, 858 m, ex *L. dalmatica* ssp. *macedonica*, leg. Toševski, DNA IT-039, voucher MAC039, haplotype pete01, (deposited in BMNH). Paratypes and depositories are listed in Appendix S2.

Diagnosis. Body robust, body length 2.5-3.7 mm [males: 2.8 ± 0.2 (2.5-3.1) $n=24$, females 3.0 ± 0.2 (2.7-3.7) $n=24$]. Scales of dorsal vestiture very fine, transparent whitish, poorly visible on both pronotum and elytra. Rostrum in lateral view moderately curved in basal half, slightly curved in apical half, distinctly longer in female (Fig. 4, –G, –H.). Second segment of antennal funicle in female 2.0X as long as wide. Pronotum blue. Elytra 1.80-1.85X as long as wide, 2.7-2.8X longer than pronotum. Penis (Fig. 5, –E, –F) with sides distinctly narrowed from base to apex. with moderately long subacute point.

Biology. Univoltine species which develops in the stems of *L. dalmatica* ssp. *dalmatica* and *L. dalmatica* ssp. *macedonica*. Biology and behaviour are similar as in *M. laeviceps* associated with *L. genistifolia*. Adults appear in the field at the beginning of March, aggregate on young growing shoots causing significant damage to the shoot points and apical leaves. The weevils copulate immediately after spring activation and first oviposition occurs in mid March, and is completed by the end of April. Females oviposit eggs singly, but lay 3 to 6 eggs at once into the lowest part of young growing shoots. Similar to *M. laeviceps*, the larva develops within short larval tunnels and newly emerged adults remain and feed in the pupal chamber until mid August. Adults overwinter in the soil close to the host plant.

Distribution. Macedonia (Pelagonia plateau), Montenegro (Boka Kotorska Bay), northern Greece (region between Florina and Prespa Lake).

Remarks and comparative notes. This species is very similar to *M. laeviceps* s.l. from which it differs by the body being more robust and usually longer, the integument of the pronotum more distinctly bluish, and the penis with longer point. Moreover the vestiture is usually formed by slightly broader scales, and is therefore generally more distinct.

Etymology. The new species is named after Dr. Peter Harris (Agriculture Canada, Lethbridge, Alberta) who has been working on the biological control of weeds since the early 1960's. Peter Harris has truly been the leader of biological control in Canada and internationally.

***Mecinus raffaeli* Baviera & Caldara sp. n.**

Type series. Holotype, male: Italy, Sicilia, Monte Etna, northern slope, Piano Provenzana, 1800 m, 15.07.2012, on *Linaria purpurea*, legg. Baviera C. & R. (BMNH). Paratypes: Monte Etna, northern slope, Monte Nero, 2000 m, 12.08.2011, on *Linaria purpurea*, leg. Baviera C., DNA IT-1480, voucher SC1480 (1, ITC); Piano Provenzana, 1800 m, 28.06.2011, on *Linaria purpurea*, legg. Baviera C. & R. (3, ITC, DNA IT-1981, 1982, 1983, vouchers SCI1982-1983); same data except 08.06.2012 (4, ITC, DNA IT-2345, 2346, 2347, 2378, vouchers SCI2345-2348 ; 10, RCC; 2, BCC).

Diagnosis Body length 2.2-2.4 mm [males: n=4, females n=6]. Scales of dorsal vestiture very fine, transparent whitish, poorly visible on both pronotum and elytra. Rostrum in lateral view moderately and regularly curved, in female moderately longer (Fig. 4, –G, –H.). Second segment of antennal funicle in female 1.5X as long as wide. Pronotum dark blue. Elytra 1.70-1.75X as long as wide, 2.50-2.60X longer than pronotum. Penis with sides distinctly narrowed from base to apex, gradually constricted at apex ending with a shorter subacute point (as in *M. laeviceps*, Fig. 5, –C, –D).

Biology. Adults of *M. raffaeli* were observed on the stems of young plants of *L. purpurea* in summer. The species seems to have a very localized distribution, as research conducted in neighboring sites has been negative (except for a single specimen found in a gall of *R. brondelii*, Brisout, 1862 at 2000 m), as well as on the eastern side of Mt Etna. In early June females have been observed on young plants of *L. purpurea* (5 to 20 cm) with their ovipositor everted in areas free from snow. On the host plant on Mount Etna, *M. raffaeli* co-lives with an extraordinarily high number of other mecinines: *M. barbarus* Gyllenhal, 1838, *M. kaemmereri* Wagner, 1927, *Rhinusa cf antirrhini* (Paykull, 1800), *R. brondelii*, *R. incana* (Kirsch, 1881) and *R. linariae* (Panzer, 1792).

Distribution. Sicily (Mount Etna).

Remarks and comparative notes. This species is distinctly geographically isolated from the other species of the group, being apparently restricted to Sicily, since *M. heydenii* is known in Italy only in the north (Caldara & Fogato, 2013). However, it is noteworthy that Caldara & Fogato (2013) examined one specimen collected in Abruzzo (central Italy) on *L. purpurea*, which they classify as *M. heydenii* and which we did not examine, belonging with high probability to *M. raffaeli*.

Although this species appears more closely related genetically to *M. heydenii*, in morphology it is distinctly more similar to *M. laeviceps* due to the shape of the rostrum and that of the penis, which

on the contrary are characters that allow to distinguish *M. raffaeli* from *M. heydenii* and *M. peterharrisi*.

Etymology. Named after the young Raffaele Baviera for his cooperation during the research of specimens on Etna Mount.

***Mecinus dorsalis* subgroup**

***Mecinus dorsalis* Aubé**

Mecinus dorsalis Aubé, 1850: 343. Bedel, 1885: 148; 1887: 311. Desbrochers des Loges, 1893: 57. Reitter, 1907: 9. Hustache, 1931: 403, 406. Hoffmann, 1958: 1267, 1273. Caldara & Fogato, 2013 in press. Caldara et al., 2013 in press.

Mecinus dorsalis var. *alpinus* Hustache, 1941: 5. Hoffmann, 1958: 1273. Caldara & Fogato, 2013 in press.

Type series. This taxon was described from a single specimen collected at Le Mans (northern France), which appears to no longer be available (Caldara & Fogato, 2013). However, we examined several specimens bearing this name, which correspond to the original description.

Synonyms. The variety *alpinus* was described from specimens collected at Col d'Allos (Basses-Alpes) at 2200 m, of which we examined the lectotype and five paralectotypes (des. Caldara & Fogato, 2013). Hoffmann (1958) synonymized this variety with *M. dorsalis*, specifying however that it might be a mountain subspecies. This synonymy was recently confirmed by Caldara & Fogato (2013).

Non-type specimens. We examined all the specimens from France reported in detail by Caldara & Fogato (2013) and moreover: **France:** Orgon, Eygalières, on *L. simplex*, DNA IT-904, voucher FRA904, haplotype dors01, leg. Toševski (1, ITC); Agny (S.M), lgt A. Hustache (1, ITC).

Diagnosis. Body length 2.1-2.6 mm [males: 2.1-2.5 mm, n = 14; females: 2.2-2.6 mm, n=10]. Scales of dorsal vestiture whitish and very visible along the middle and sides of the pronotum and on the elytral interstriae. Rostrum in lateral view distinctly and abruptly curved in basal half, weakly narrowed in apical half, in female slightly longer. Second segment of antennal funicle in female 1.5X as long as wide. Pronotum black, shining. Elytra black, somewhat shining; 1.70-1.80X

as long as wide, 2.60-2.80X longer than pronotum, on elytral interstriae with recumbent scales. Penis as in *M. heydenii* but with longer and acute point.

Biology. Univoltine species which develops on *Linaria simplex*. Adults appear at the beginning of March. Females oviposit early in the spring (March-April). Oviposition takes place inside apical part of the young shoots. Oviposition induces a somewhat elongated apical gall, while the attacked shoot is stunted in growth. Larva develops inside gall tissue. *Linaria repens* (L.) Mill., *L. polygalifolia* Hoffmanns and Link, *L. saxatilis* (L.) Chaz. and *L. triornithophora* (L.) Willd. are quoted as potential host plant species (Hoffmann 1958), but host association with regard to larval development of *M. dorsalis* remains to be confirmed.

Distribution. France.

Remarks and comparative notes. Due to the shape of the rostrum, which is strongly curved at base, this species is morphologically similar to *M. heydenii*, from which it differs by the completely black integument without metallic reflection, and the distinctly longer point of the penis. It is very similar to *M. bulgaricus* from which it differs by a more curved rostrum at base, and further nearly straight and the hair-like scales of the elytral interstriae that are almost completely recumbent.

***Mecinus bulgaricus* Angelov**

Mecinus bulgaricus Angelov, 1971: 115. Arzanov, 2000: 1087. Caldara & Fogato, 2013 in press

Type series. Angelov (1971) described *M. bulgaricus* from a unique female specimen collected at Harmanli (eastern Rhodopi, south-eastern Bulgaria) on April 16th 1970. Arzanov (2000) reported to have examined this specimen, received from MZHF where Angelov's collection was deposited after Angelov's death. However he refers that this specimen was collected on April 23rd 1971 and does not bear a label indicating that this is the holotype, on the contrary of all other taxa described by Angelov. He concluded that this taxon is synonymous with *M. heydenii* and this opinion was accepted, although doubtfully, by Caldara & Fogato (2013). Actually this specimen is not the holotype of *M. bulgaricus* as the date of collection clearly demonstrates. According to Arzanov (2000) this specimen has dark blue elytra, whereas in the original description it is clearly reported that *M. bulgaricus* has black integuments with bronze reflections on the pronotum as in *M. aubei* from northern Africa. Unfortunately, it is currently impossible to locate both the specimen examined by Arzanov and the holotype of *M. bulgaricus* at MZHF (Muona and Silfverberg, pers. comm.). However, we examined four specimens with black elytra, checked as *M. dorsalis* Aubé by

Caldara & Fogato (2013), three of which were collected in Bulgaria and one in Greece (see detailed data below), corresponding perfectly to Angelov's description. Moreover the specimen from Strandzha-Kosti (Bulgaria, south of Primorsko) was collected by Angelov and identified by him as *M. bulgaricus*, whereas another of these specimens was recently collected by Toševski at the type locality of *M. bulgaricus*.

Non-type specimens. Bulgaria: Strandzha-Kosti, 20.04.1974, leg. Angelov (1, MMC); Jasnaja Poljana, Burgas (1, PBC); Rodopi, Severoiztočni, Kalojanci, 16.VI.1982, Košťál leg. (1, JFCH); Harmanli, 12.04.2012, feeding on *L. genistifolia*, voucher BG2187, haplotype bulg01, leg. Toševski (1, ITC). **Greece:** Gerakini, Halkidiki (1, DEI).

Diagnosis. As *M. dorsalis* except: body length 1.9-2.3 (males n = 2; females = 2). Rostrum more regularly arcuate from base to apex, less abruptly curved at base. Elytral interstriae with scales slightly but distinctly raised from integument.

Biology. The host plant of *M. bulgaricus* is unknown. Our single specimen was collected from the type locality (Harmanli, south-east Bulgaria), feeding upon *L. genistifolia* ssp. *Sofiana*, which is the known host plant of *M. laeviceps meridionalis*.

Distribution. East Bulgaria and Greece (Aegean coast).

Remarks and comparative notes. It is here confirmed that the specimens from Bulgaria and Greece, which were doubtfully placed together *M. dorsalis* from France by Caldara & Fogato (2013), actually belong to a different species on the basis of the genetic data. However these two species are very similar morphologically differing each other only by the shape of the rostra and the disposition of the elytral vestiture.

Key to the species of *Mecinus heydenii* species complex

1. Integument of elytra entirely black, at most with weak bronze reflection. Point of penis long.....2
- Integument of elytra always more or less bluish with metallic reflection. Point of penis moderately to slightly long.....3
2. Rostrum very strongly curved at base, then weakly curved to apex. Scales of elytral interstriae nearly recumbent..... *M. dorsalis*

- Rostrum distinctly but more regularly curved from base to apex, less abruptly curved at base.
- Scales of elytral interstriae slightly but distinctly raised..... *M. bulgaricus*
- 3. Rostrum in lateral view very arcuate from base to apex, strongly curved especially before antennal insertion (Figs.). Penis with sides slightly narrowed from base to apex, ending in a distinctly constricted, moderately long, subacute point..... *M. heydenii*
- Rostrum in lateral view moderately arcuate from antennal insertion to apex. Penis with sides more narrowed from base to apex, ending in a short to moderately long point.....4
- 4. Elytra 1.70-1.75X as long as wide, 2.50-2.60X longer than pronotum. Second segment of antennal funicle in female 1.5X as long as wide..... *M. raffaelli*
- Elytra 1.80-1.85X as long as wide, 2.70-2.80X longer than pronotum. Second segment of antennal funicle in female 2.0X as long as wide.....5
- 5. Pronotum usually black with distinct bronze reflections. Scales of dorsal vestiture whitish and well visible along middle and sides of pronotum and on elytral interstriae. Penis ending in a short point..... *M. laeviceps*
- Pronotum usually blue. Scales of dorsal vestiture very fine, transparent whitish, poorly visible on both pronotum and elytra. Penis ending in a moderately long point..... *M. peterharrisii*

Discussion

The study of *M. heydenii* was primarily undertaken because of its potential use as a biological control agent for invasive toadflaxes in North America. As such, the taxonomical and nomenclatural stabilization of taxa within the *M. heydenii* species complex is of great importance for the applied study of these potentially beneficial weevils. According to previous morphological studies (Arzanov, 2000; Caldara & Fogato, 2013) our initial hypothesis was that *M. heydenii* was a single morphologically variable species with some synonyms as *M. laeviceps* and *M. bulgaricus*. Analyses of mitochondrial COII gene sequences revealed consistent host-associated genetic structure among sampled populations of *M. heydenii* s.l. from different host-plant species from the genus *Linaria*. In Bayesian, maximum parsimony, neighbor-joining and statistical parsimony analyses, weevil COII haplotypes retrieved from a particular host plant species cluster together with high bootstrap support, showing substantial genetic divergences from 6.0-14.6% among species, with divergence within the group estimated to have initiated approximately 8 mya.

In contrast to previous conclusions based on morphology alone (Hoffmann 1958, Smreczyński 1976, Lohse & Tischler, 1983, Arzanov, 2000, Caldara & Fogato, 2013), our study assigned full

species status to six host associated weevils populations: *M. heydenii* associated with *Linaria vulgaris*, *M. laeviceps* with *L. genistifolia* and *L. corifolia*, *M. raffaelli* with *L. purpurea*, *M. peterharrisi* with *L. dalmatica*, *M. dorsalis* with *L. simplex*, while the host plant of *M. bulgaricus* still remains unknown. Additionally, conclusions of host plant associated genetic differentiation were supported by substantially higher offspring survival of the two *Mecinus* species tested, *M. heydenii* and *M. laeviceps* on their respective host-plant species. Oviposition of *M. heydenii* on *L. genistifolia* (the host plant species for *M. laeviceps*) was followed by extremely strong hypersensitive stem reactions enabling larval development of *M. heydenii*, while *L. vulgaris* was not a suitable host plant to support larval development of *M. laeviceps*. These results strongly suggest the existence of highly specific interactions between weevils and their host plant species that could be driving co-evolutionary adaptations of *Mecinus* taxa to their respective host plants, facilitating genetic differentiation of host associated populations.

This study demonstrates how taxonomic work integrated with accurate biological and genetic data can improve the delimitation of species boundaries. It is noteworthy that the re-examination of the material recently used by Caldara & Fogato (2013) for their morphological revision of the genus *Mecinus*, revealed that several characters (i.e. the shape of the rostrum, the colour of the integument and the density of the vestiture), previously considered “variable” on the basis of museum specimens without data except those on locality of collection, were actually representative of cryptic species. Within the Mecinini, it is a common feature that many species differ from each other only slightly, by a few characters such as the shape of the rostrum and the disposition of the dorsal vestiture (e.g. *Mecinus variabilis* vs *M. caucasicus*, *Rhinusa pilosa* vs *R. brondelii*, *Rhinusa antirrhini* vs *R. dieckmanni*, *Gymnetron rostellum* vs *G. aper*, *Mecinus janthinus* vs *M. janthiniformis* etc.) (Caldara, 2008; Caldara et al., 2008; Caldara et al., 2010; Caldara & Fogato, 2013; Toševski et al, 2011) showing elements of cryptic speciation. Mopper (1996) suggested that the close association between endophagous insects and their host plants may augment the selection pressure imposed by the host, which may lead to diversification, specialization and ecological speciation. The occurrence of genetically distinct host associations in phytophagous insects has been well documented (Feder et al., 1988; Via, 1999; Via et al., 2000; Hernandez-Vera et al., 2010), and hybridization among congeneric plant species, such as occurs within the genus *Linaria*, may create conditions that enhance host shifting of endophagous insects and subsequent ecological divergence (Berlocher and Feder, 2002, Funk et al., 2002). With regard to their distribution, the four taxa belonging to *M. heydenii* group, studied here, follow that of the host plant distribution, although data for the western Palaearctic are still lacking.

On the basis of the genetic data, the species studied here appear to form two monophyletic assemblages within the *M. heydenii* group as considered by Caldara et al. (2013), i.e. the *M. heydenii* and the *M. dorsalis* subgroups. This conclusion is morphologically supported by a single character state represented by the different colour of the integument, which is partly blue or completely black respectively. These two groups diverged from each other approximately 8.1 Ma (95% HPD 5.1-10.8) placing their initial divergence time within a period of dramatic geological events in southern Europe during the late Miocene (11.06-5.33 Ma). Furthermore, the estimated divergence times which lead to subsequent speciation within these two groups coincides with the period just after Messinian salinity crisis, dated between 5.6-5.3 million years ago (Krijgsman et al., 1999). This period resulted in a broad range of land connections (de Jong, 1998) and the dispersal of floristic and faunistic elements across the Mediterranean basin (Montreuil, 2008) prior to rapid inundation during the Zanclean flood (Garcia-Castellanos et al., 2009). Consistent with these events, our results indicate the existence of biogeographical vicariations inside both lineages of species. Isolation of ancestral populations forced by vicariant host plant associations, may have lead to another period of speciation with estimated timing of 3.4 Ma for *M. heydenii* and *M. raffaeli*, and 2.75 Ma for *M. laeviceps* and *M. peterharrisi*, respectively. It is noteworthy that only two species, *M. heydenii* and *M. laeviceps*, possess broad geographic distributions which extend beyond Messinian events within Mediterranean basin, while *M. raffaeli* and *M. peterharrisi* are species with restricted Mediterranean distributions inhabiting confined geographic areas which appear to be highly endemic e.g. Sicily and Balkan (Montreuil, 2008). The most recent genetic segregation was recorded within *M. laeviceps* populations, dated about 0.75 and 0.51 Ma, showing genetic divergence between three geographically disjunct populations, two of which are close to Mediterranean regions, suggesting ongoing speciation mediated by geographic isolation (ssp. *meridionalis*) and host shift (ssp. *corifoliae*).

Knowledge about the evolutionary history of a particular focal group of taxonomic uncertainty, as well as their field host associations, represents an important contribution to the validation of taxon boundaries and thus their potential as candidate agents in biological control programs. We have revealed the existence of cryptic speciation processes corresponding to clear host associated genetic divergence within the *M. heydenii* species complex, where assigned taxa possess explicitly monophagous ecological host ranges in regard to their respective host plants from the genus *Linaria*. By increasing taxon sampling and using integrative taxonomic approaches in species delimitation, our study has increased the number of weevil species within the *M. heydenii* species

complex, and additionally, increased the number of weevil species that can be considered as potential biocontrol agents for the control of invasive toadflaxes of Euro-Asian origin. The biological control programs in North America against invasive toadflaxes are presently focused on *L. vulgaris* and *L. dalmatica*. In our study, at least four weevil species or subspecies from *M. heydenii* species complex could potentially be assigned for this purpose, *M. heydenii*, *M. laeviceps* ssp. *laeviceps*, *M. laeviceps* ssp. *meridionalis* and *M. peterharrisi*.

Conclusions

This study provides support for two main species subgroups within the *M. heydenii* complex (*M. heydenii* and *M. dorsalis* subgroups) and emphasizes the utility of phylogenetic and evolutionary data for resolving species boundaries among morphologically cryptic taxa. The results of this study provide new evidence regarding species limits within taxa inside the *M. heydenii* species complex, revealing genetically well-differentiated taxa with cryptic phenotypic characteristics. Six taxa are assigned full species status. In accordance with previous investigations, the full species status of *M. heydenii* and *M. dorsalis* is confirmed, while species status of *M. laeviceps* and *M. bulgaricus* is recovered. Two new taxa are described, *M. raffaelli* and *M. peterharrisi* associated with *L. purpurea* and *L. dalmatica*, respectively. In addition, within *M. laeviceps*, two subspecies are described in association with *L. genistifolia* (ssp. *meridionalis*) and with *L. corifolia* (ssp. *corifoliae*), both showing measurable genetic divergence from the nominal subspecies. All taxa from the *M. heydenii* species complex are highly specific in regard to host plant species used from within the genus *Linaria*.

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