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Retrospective use of integrative taxonomy in classical biological control: The unintentional introduction of the weevil *Rhinusa dieckmanni* to North America

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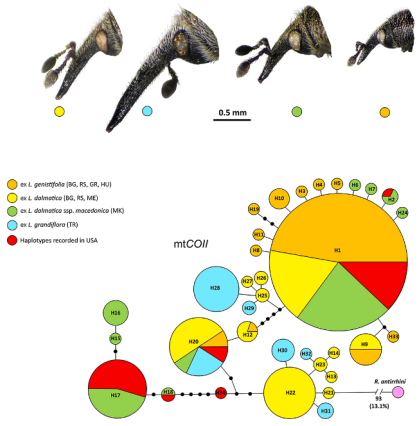
HIGHLIGHTS

- Retrospective use of integrative taxonomy in classical biological control.
- Unintentional NA introduction of *Rhinusa dieckmanni.*
- Genetic and morphological evaluation of species status.
- Absence of genetic clustering between weevil populations on different *Linaria* spp.

GRAPHICAL ABSTRACT



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ABSTRACT

A seed-feeding weevil introduced to North America (NA) as a biological control agent of the invasive toadflax *Linaria dalmatica* (L.) Mill., identified then as *Gymnetron antirrhini* "Dalmatian host race" and subsequently confirmed as established, was revealed through our study to be a separate species, i.e., *Rhinusa dieckmanni* (Behne) (Coleoptera: Curculionidae). This weevil species was presumed to be endemic in its native range, with a distribution restricted to Mount Rila in southwestern Bulgaria. We conducted a comprehensive study of seed-feeding weevils associated with *L. dalmatica*, *L. dalmatica* ssp. *macedonica* (Griseb.) D.A. Sutton, *L. genistifolia* (L.) Mill., and *L. grandiflora* Desf. across a broad geographic area of their native range. Those results revealed that all four host plants were used by *R. dieckmanni* and thus the native geographic range of the species is wider than

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expected, encompassing the Balkans and the Anatolian Plateau. Our observations suggest that phenotypes of this weevil are highly variable and dependent on the seed capsule size of the *Linaria* host population. The haplotype network based on mitochondrial *COII*, *16S* genes, and nuclear *EF 1-α* gene genealogy confirmed the conspecific nature of geographically distant weevil populations, that is, *R. dieckmanni* phenotypes utilizing *L. genistifolia*, *L. dalmatica*, and *L. grandiflora* for larval development. Specimens collected from *L. dalmatica* in the northwestern USA shared the same haplotypes as samples from *L. dalmatica* ssp. *macedonica* in southwestern North Macedonia, supporting the known introduction history of the North American population. Females from these populations have relatively short rostrums, which may limit their reproductive success on North American invasive *L. dalmatica* with larger seed capsules.

1. Introduction

Classical biological control of exotic, invasive *Linaria* (toadflaxes) species in North America (NA), initiated in the early 1960s, utilizes insects associated with three plant species or forms in the native range: *L. vulgaris* Mill., *L. genistifolia* (L.) Mill., and *L. dalmatica* (L.) Mill. (De Clerck-Floate and Turner, 2013). Following host-specificity screening and regulatory approval, five agents were intentionally introduced, primarily to western North America, over a thirty-year period (Sing et al., 2022). Introduced agents included a foliar-feeding moth, *Calophasia lunula* (Hufnagel) (Lepidoptera: Noctuidae), introduced in 1965; two root-boring moths, *Eteobalea serratella* (Treitschke) and *E. intermediella* (Riedl) (Lepidoptera: Cosmopterigidae), introduced in 1995; a *Gymnetron* root-galling weevil, now known as *Rhinusa linariae* (Panzer) (Coleoptera: Curculionidae), introduced in 1995; and a stem-mining weevil, *Mecinus janthinus* Germar (Coleoptera: Curculionidae), introduced in 1991 (De Clerck-Floate and Harris, 2002). *Mecinus janthinus,* intentionally introduced to control *L. vulgaris*, was subsequently determined using integrative taxonomy to be a complex of two cryptic species, with the second one, *Mecinus janthiniformis* Toševski & Caldara, predominantly associated with *L. genistifolia* and *L. dalmatica* (Toševski et al., 2011; 2018). In addition, three toadflax feeding insect species of European origin are thought to have been adventitiously introduced from the beginning of the 20th century (Smith, 1959). These include the flower-feeding beetle, *Brachypterolus pulicarius* (Linnaeus) (Coleoptera: Kateridae), and two *Gymnetron* seed-capsule feeding weevils, now known as *Rhinusa antirrhini* (Paykull) and *R. neta* (Germar), (Coleoptera: Curculionidae) (Sing et al., 2022).

The presence of *R. antirrhini* (previously treated as *Gymnetron antirrhini*) in North America was first recorded in 1909 (Pierce, 1919), in association with *L. vulgaris* and with a narrow-leaved form of *L. dalmatica* (Smith, 1959). Individuals collected in 1957 from a Belleville (Ontario, Canada) population in association with *L. vulgaris* were used for introductions made in the western Canadian provinces Saskatchewan and Alberta (Smith, 1959). *Rhinusa antirrhini* is now widely distributed across Canada and the USA (Sing et al., 2016).

Unlike *R. antirrhini* (ex *L. vulgaris*), which was accidentally introduced to NA, the NA introduction of *R. antirrhini* collected in the native range from *L. dalmatica* was planned and intentional. A "Dalmatian host race" of *R. antirrhini* was collected from *L. dalmatica* ssp. *macedonica* (Griseb.) distributed in the southwestern part of North Macedonia, from several locations between the towns of Prilep and Resen (Pelagonian mountain plateau) (Groppe, 1992). It was first released in Canada in 1993 (De Clerck-Floate and Harris, 2002) and in the U.S. states of Montana and Wyoming in 1996 (Winston et al., 2023). Shortly after, an intensive debate took place regarding the species limit between *R. antirrhini* populations associated with *L. vulgaris*, i.e., *R. antirrhini* s. str., and those associated with *L. dalmatica*. Sing et al. (2005) reported the possibility that the *L. dalmatica*-adapted host race of *R. antirrhini* was likely a separate and unnamed species due to significant differences in mitochondrial DNA profiles. Nonetheless, populations associated with *L. dalmatica* were not intensively screened pre-release, because they were assumed to be a host race of an accidentally introduced but long-established species, *R. antirrhini* (Groppe, 1992). However, despite multiple introductions of *R. antirrhini* "Dalmatian host race" during the 1990s in Canada and the USA, establishment in North America was sporadic and population numbers remained low (Sing et al., 2016).

Literature on *R. antirrhini* "Dalmatian host race" is generally associated with studies related to the biological control of invasive toadflaxes in North America (Sing et al., 2016). A taxonomic review of the *R. antirrhini* species complex known to be associated with *L. dalmatica* and its closest relatives is conspicuously lacking. This would include a key species, described under the name *Gymnetron* (*Rhinusa*) *dieckmanni,* from southwestern Bulgaria. This species was described by Behne (1988), who reported *L. dalmatica* as a host plant of the newly recognized taxon. According to the original description, a primary distinguishing characteristic of *R. dieckmanni* is the long rostrum in females, which is significantly longer than in the closely related species *R. antirrhini* associated with *L. vulgaris*, or the fruit-gall weevil *R. smreczynskii* (Fremuth), the latter currently synonymous of *R. florum* (Rübsaamen) (Caldara, 2008), and associated with *L. genistifolia* and *L. dalmatica*. According to available records, the distribution of *R. dieckmanni* is restricted to the type locality on Mount Rila, and thus the species is regarded as a Balkan endemic with limited distribution.

Previous efforts to define the natural host range of *Linaria*-associated weevils across the western Palearctic consistently revealed the presence of cryptic species, with genetic segregation likely driven by strict host plant association (Toševski et al., 2011; 2014; 2015). Pronounced host-associated genetic differentiation was similarly recorded within native range *R. antirrhini*, with clear separation between specimens associated with *L. vulgaris* (=*R. antirrhini* s.str.) and those associated with *L. genistifolia* and *L. dalmatica* (Hernández-Vera et al., 2010). Contemporaneous efforts to collect and include in that analysis specimens of *R. dieckmanni* from the type locality (Rilski Monastery, Mount Rila, Bulgaria) were unsuccessful. The first objective in the present study was therefore to perform a comprehensive evaluation of the identity of the *R. antirrhini* "Dalmatian host race" intentionally introduced in the 1990s to North America, using morphological and population genetic analyses. Specimens associated with *L. dalmatica* and *L. genistifolia* of Balkan origin, including *R. dieckmanni* from the Mount Rila type locality, as well as from *L. grandiflora* of Anatolian origin, were compared with specimens obtained from *L. dalmatica* in North America. Secondly, probable reasons are discussed for the low representation of the Dalmatian host race of the weevil in North American samples, compared to its relative abundance in the native range.

2. Material and methods

2.1. Insect sampling

Between 2006 and 2018, weevils in the genus *Rhinusa* were sampled from Serbia, Hungary, Montenegro, North Macedonia, Greece, Bulgaria, Turkey, and the USA. The majority of adults were reared from larvae infesting field collected seed capsules of three *Linaria* species, *L. genistifolia* from Serbia, Hungary, Bulgaria, North Macedonia and Greece; *L. dalmatica* from Serbia, Bulgaria, Greece, Montenegro, North Macedonia (ssp. *macedonica*) and the USA; and *L. grandiflora* from Turkey (Fig. 1). The emerged adults used for morphological and genetic

analyses were stored in 96% ethanol at + 4 °C, and labeled with collection location, date, and host plant. Details regarding native range location and host plant affiliation of each analyzed specimen are presented in Supplementary Table S1; the same details for USA specimens are provided in Table S2. In addition, eight localities in the northwestern USA where a total of 396 *Rhinusa* spp. specimens were collected from *L. dalmatica* are listed in Supplementary Table S3.

2.2. Morphological study

The collected weevils were examined using a Leica MS5 stereomicroscope. Representative specimens were photographed using a Leica 165C multifocus system. The weevils were separated into seven groups (i.e., populations, in a broader sense) according to their geographical origin and host plant affiliation: 1) Bulgaria – type locality/*L. dalmatica*, 2) Turkey/*L. grandiflora*, 3) North Macedonia/*L. dalmatica* ssp. *macedonica*, 4) Eastern Serbia/*L. dalmatica*, 5) Eastern Serbia/*L. genistifolia*, 6) central Bulgaria/*L. genistifolia*, 7) central Serbia/*L. genistifolia*. Measurements taken from 30 randomly selected males and females per group included length of body from the anterior margin of pronotum (head and rostrum excluded) along the midline to the apex of the elytra, and the length of the rostrum measured in lateral view from the apex to the anterior margin of the eye. A one-way ANOVA followed by a Tukey's HSD test was used to compare means of the obtained measurements according to location and host plant affiliation of the respective weevil populations.

2.3. Molecular study

Molecular genotyping included weevil specimens collected from different *Linaria* species in southeastern Europe, Turkey (Fig. 1), and northwestern USA. Individual weevils were punctured between the 2nd and 3rd thoracic sternites, and total DNA was extracted from whole specimens using the QIAGEN DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following the DNA extraction all specimens were prepared as dry voucher specimens. The complete mitochondrial cytochrome oxidase subunit II gene (*COII*) was analyzed as the primary marker for the molecular identification, characterization, and differentiation of the weevil specimens; consequently, all specimens utilized in the molecular genetics study were subjected to *COII* gene typing. The *COII* amplification was carried out according to a previously published protocol (Hernández-Vera et al., 2010). Mitochondrial *16S* ribosomal RNA (*16S*) and nuclear elongation factor-1α (*EF-1α*) genes were analyzed in a subset of weevil specimens selected based on their *COII* haplotype to cover the majority of the diversity and to supplement information on the species' variability. The *16S* and *EF-1α* genes were amplified following previously described protocols (Toševski et al., 2015).

Pairwise distances between haplotypes associated with specific host plants were analyzed using the p-distance model integrated in MEGA5 (Tamura et al., 2011). Gene genealogies of *COII*, *16S* and *EF-1α* were inferred using TCS, version 1.21 (Clement et al., 2000), and haplotype networks were constructed using statistical parsimony with a confidence limit of 95%. All obtained haplotype sequences were deposited in the NCBI GenBank database under the following accession numbers: MW837172–MW837210 for *COII*, ON920817–ON920823 for *16S*, and ON934913–ON934919 for *EF1-α* gene.

To assess hierarchical levels underlying variability in the mt*COII* gene, we performed an analysis of molecular variance (AMOVA) using Arlequin 3.5.1.2 (Excoffier and Lischer 2010). *Rhinusa dieckmanni* populations were grouped according to a) their host plant usage, i.e., *L. genistifolia*, *L. dalmatica,* and *L. grandiflora*, and b) affiliations with geographical region. Populations with low sample sizes were omitted from the analyses.

3. Results

3.1. Insect sampling

Overall, more than 600 native range *Rhinusa* specimens were collected, of which 180 adults from 37 localities in southeastern Europe and Turkey were included in the molecular study. Analyses included weevils collected from *L. genistifolia* (n = 47), *L. dalmatica* (n = 68), *L. dalmatica* ssp. *macedonica* (n = 32), and *L. grandiflora* (n = 33) (Table S1). North American *Rhinusa* were all collected from *L. dalmatica* resulting in 396 specimens of which only 24 were *R. antirrhini*

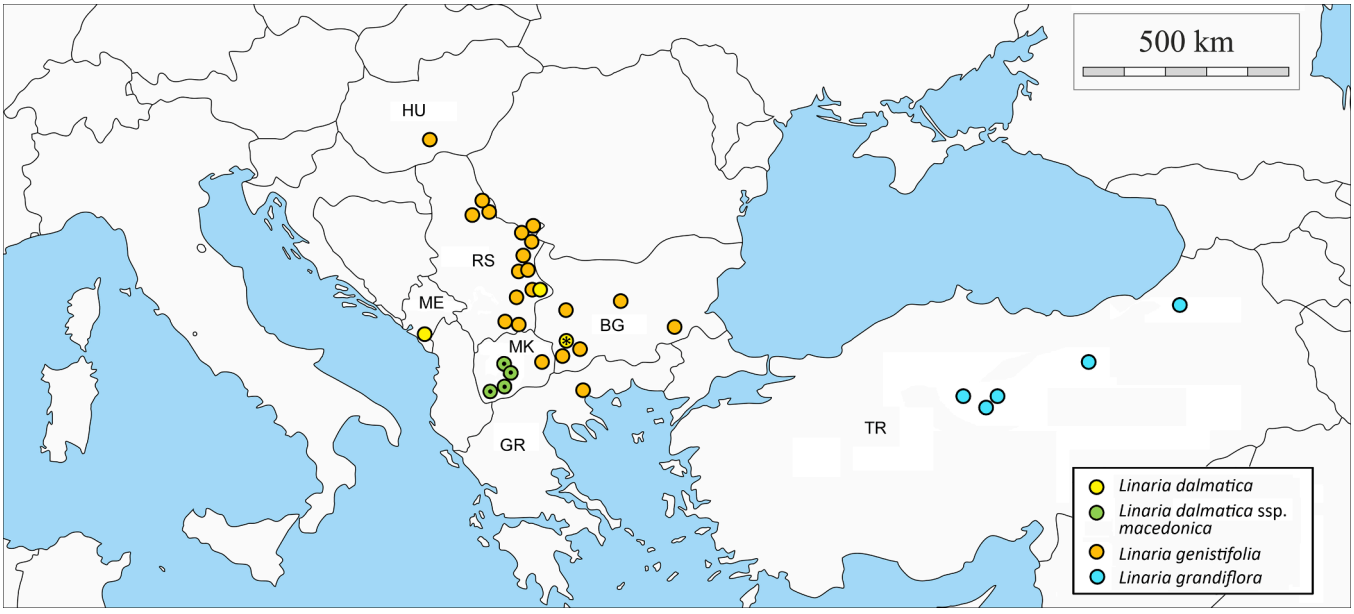

Fig. 1. Location of sampling sites for *Rhinusa* specimens collected from different *Linaria* species in Europe. The asterisk inside the circle marks the type locality of *Rhinusa dieckmanni* (Mount Rila, Bulgaria). Dots inside circles indicate locations of *L. dalmatica* ssp. *macedonica* from which *Rhinusa antirrhini* "Dalmatian host race" was introduced to North America. Labeled countries with two letter code represent current distribution of *R. dieckmanni*. For details regarding locations see Supplementary Table S1.

"Dalmatian host race" (6.1%) while 372 specimens were *R. neta* (93.9%). *Rhinusa antirrhini* "Dalmatian host race" specimens were obtained from only three of nine collection sites, which clearly demonstrates the disproportional abundance of the two *Rhinusa* species, and limited distribution of the former species, even where it was first and repeatedly released in the USA. Further, the *R. antirrhini* "Dalmatian host race" was only confirmed from specific locations where it was known to have been released in the past: Coxcy Gulch, North Fork Little Boulder River, and Mount Helena (Table S3).

3.2. Morphological analysis

The weevil specimens exhibited a high degree of variation in rostrum length (Fig. 2) and body size (Fig. 3). This variability was recorded at intra- and inter-population levels. The observed array of phenotypes, with regard to range in rostrum and body length (Fig. 4), was observed both within and between geographical groups, and in association with three species and one subspecies of toadflax. Significant differences in rostrum length in females ($F_{(6,\ 203)} = 359.7$, $p < 0.05$) and males ($F_{(6,\ 203)} = 81.0$, $p < 0.05$) were recorded between all analyzed populations, including *R. dieckmanni* collected from the type locality (Mount Rila, Bulgaria). However, post-hoc comparison of the means after one-way ANOVA analysis showed no significant differences ($p > 0.05$) between populations from North Macedonia associated with *L. dalmatica* ssp. *macedonica*, the Eastern Serbia population associated with *L. dalmatica*, and the Eastern Serbia populations associated with *L. genistifolia,* with the last two commonly recorded in syntopy. Significant differences in body length were also observed in females and in males, $F_{(6,\ 203)} = 135.8$, $p < 0.05$ and $F_{(6,\ 203)} = 100.6$, $p < 0.05$, respectively. Recorded phenotypic plasticity in seed-feeding weevils associated with *L. genistifolia*, *L. dalmatica* and *L. grandiflora* demonstrated that traditional morphological characters alone are not reliable for defining stable differences among the analyzed populations. Nevertheless, the most prominent difference recorded within weevil populations was the length of the rostrum in the females, which ranged from 0.3 to 1.4 mm (Table S4), a character strongly influenced by weevil body size, and the size of the seed capsule in which larval development was completed (Figs. 4 and 5).

Recorded variability in the weevils collected from 37 populations in association with L. dalmatica, L. genistifolia and L. grandiflora in southeastern Europe and Turkey strongly suggests that morphological variation in the weevil populations coincides with variability in seed capsule size of the host plants.

3.3. Molecular analysis

Complete mt*COII* gene was 681 bp in all of 204 sequenced specimens, including those collected in North America. The sequence analysis yielded 34 haplotypes, with a total of 39 (5.7%) polymorphic nucleotides, of which 18 (2.6%) were parsimony informative. Average pairwise distance between recorded haplotypes was 1% with maximum in-group genetic distance of 2.3% (uncorrected, data not shown).

The haplotype network based on statistical parsimony showed no reticulations between *COII* haplotypes (Fig. 6a). Mutual connections observed between haplotypes did not support the existence of a clear geographical or host plant associated pattern of segregation. The most frequent haplotype, H1, was recorded almost equally in the specimens originating from *L. genistifolia* and *L. dalmatica*, while another three widely distributed haplotypes (H9, H12 and H20) were also shared between these two plants. Moreover, haplotype H20 was associated with specimens collected from *L. dalmatica* (type locality of *R. dieckmanni,* Mount Rila, Bulgaria), *L. dalmatica* from Eastern Serbia (Pirot), *L. genistifolia* also from Eastern Serbia (Tresibaba, Svrljig Mountains) and *L. grandiflora* from Turkey, showing a wide distribution of this haplotype, from eastern Anatolia to the central Balkans.

A total of five haplotypes were recorded from specimens associated with *L. dalmatica* from the *R. dieckmanni* type locality, expressing average pairwise distance of 0.7% and a maximum genetic distance of 1%. In the weevil population sampled from Eastern Serbia *L. dalmatica,* a total of 8 haplotypes were recorded with average pairwise distance of 0.7% and a maximum genetic distance of 1.5%. Average pairwise distance of 1.2% and maximum genetic distance of 2.3% was recorded in the nine haplotypes from the weevils associated with *L. dalmatica* ssp. *macedonica.* For weevils associated with *L. grandiflora* and *L. genistifolia*, average pairwise distance and maximum genetic distance was 0.8 and 1.2%, and 0.6 and 1.3 %, respectively.

Overall, the gene genealogy shows high diversity across the distribution range of this species, with clear signs of gene flow between populations grouped according to host plant usage and distinct geographic regions (e.g., haplotypes H1, H9, H12 and H20). Furthermore, haplotype H20 was recorded in specimens with different phenotypes following rostrum and body length, depending on the toadflax

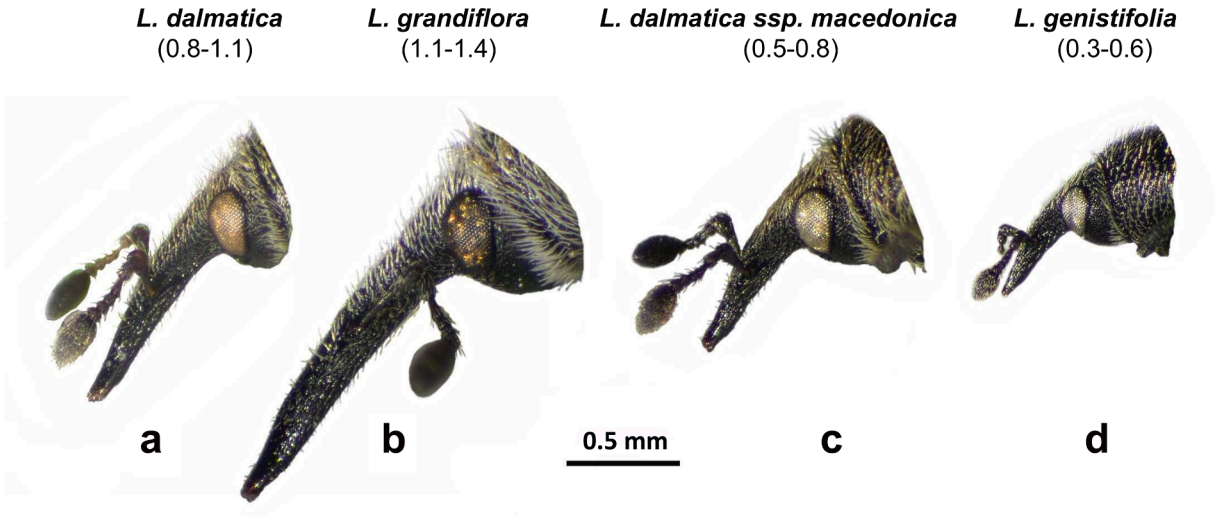

Fig. 2. Variability of rostrum length in *Rhinusa* weevils females utilizing different *Linaria* species and populations for oviposition and development: a) ex *L. dalmatica*, Bulgaria, Mount Rila (type locality of *R. dieckmanni*); b) ex *L. grandiflora*, Turkey, Kırşehir; c) ex *L. dalmatica* ssp. *macedonica,* North Macedonia, Prilep (founder population, *Rhinusa antirrhini* "Dalmatian host race" intentionally introduced to North America); and d) ex *L. genistifolia*, Bulgaria, Bansko. Rostrum length range is given in brackets (Appendix A, Table S4).

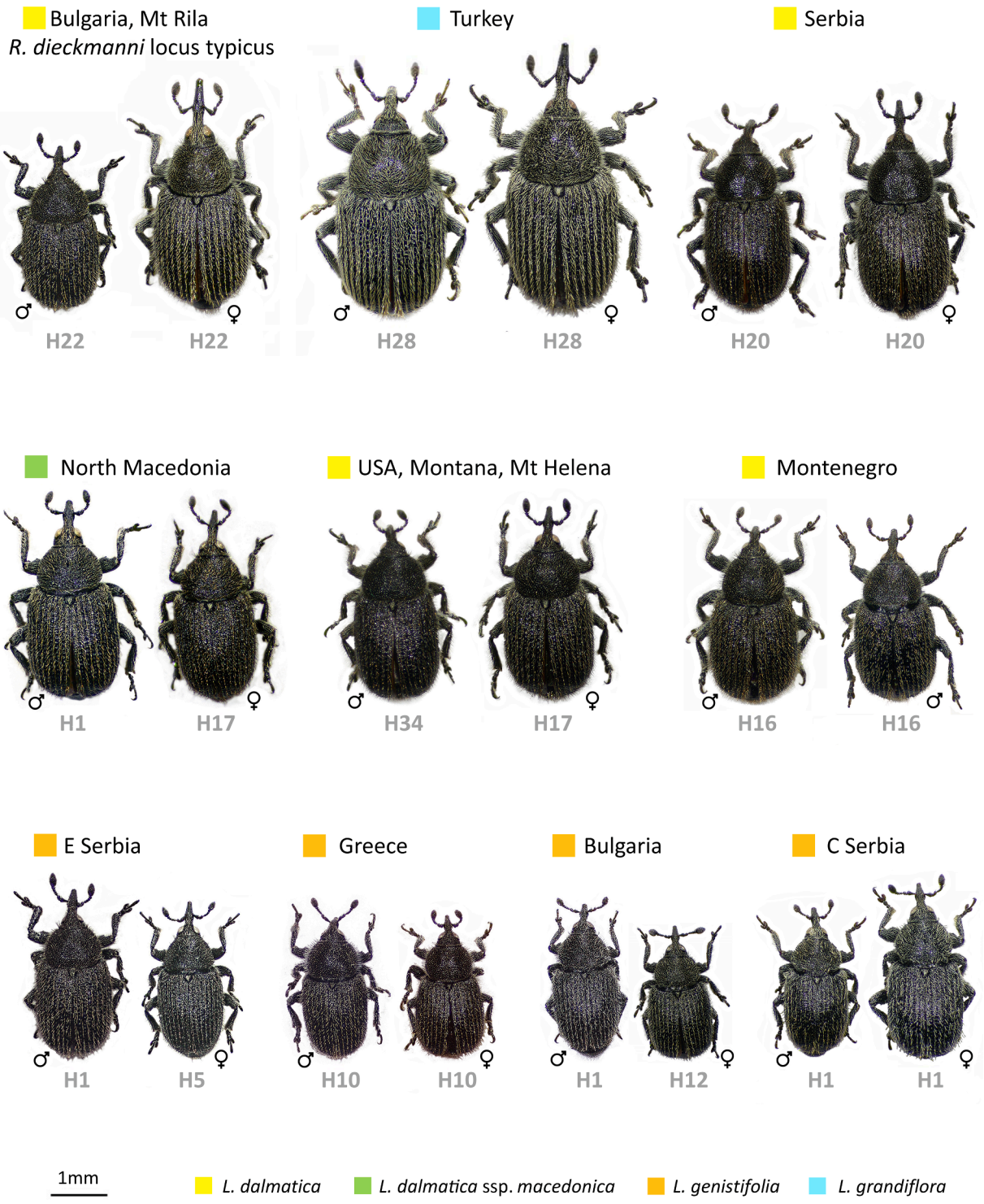

Fig. 3. Variability in body size among *Rhinusa* weevils utilizing seed capsules of different *Linaria* species and populations for larval development. Geographic and ecological (host-plant) origin for each specimen is given above images. The color of squares in the figure legend ascribes host plant association at collection sites of the depicted weevil populations. Mitochondrial *COII* gene haplotype affiliation of each presented specimen is denoted directly below in grey font. Mean body length and range is given in Appendix A, Table S4.

phenotype utilized for larval development, i.e., with large or small seed capsules. A wide geographic range is also recorded for haplotype H22; it was recorded in the type locality of *R. dieckmanni* and in Eastern Serbia, in association with *L. dalmatica.* From this haplotype, a total of four haplotypes are derived by a single nucleotide substitution, two associated with *L. grandiflora* (Turkey), and two associated with *L. dalmatica* from Eastern Serbia and Mount Rila (Bulgaria). Haplotype H32 which is associated with *L. grandiflora* (Alişar, Turkey) is also grouped in this subcluster (Fig. 6a).

A total of six haplotypes were recorded in 24 sequenced specimens from North America (Fig. 6). Haplotypes H2, H17 and H18 confirmed their affiliation with specimens originating from *L. dalmatica* ssp. *macedonica*, which served for several years as the primary source for introductions to North America. H1, the most frequent and widely

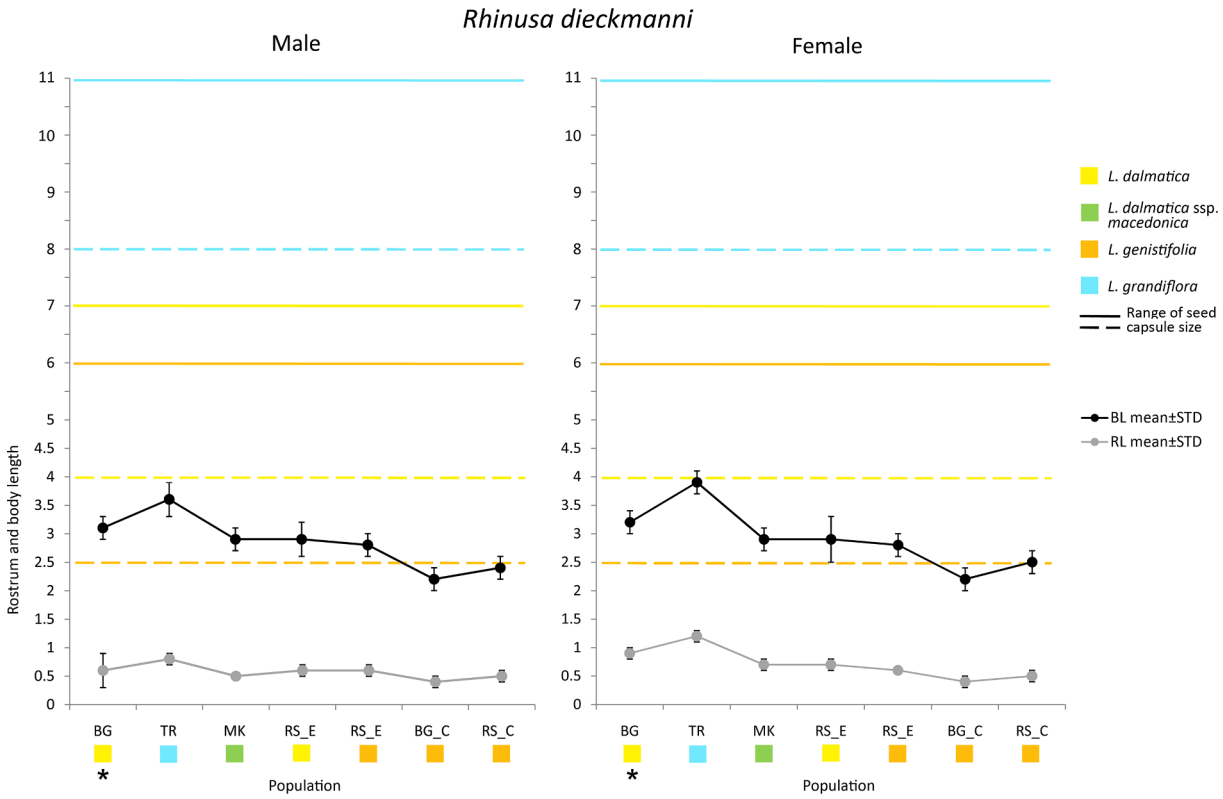

Fig. 4. Variation in mean (±STD) body length (BL, black) and rostrum length (RL, grey) of molecularly confirmed males and females of *Rhinusa dieckmanni* s.str. (population from type locality, denoted with asterisk) and populations of different geographic origin associated with diverse *Linaria* spp. populations. Geographic origin of sampled *R. dieckmanni* populations is designated as follows: BG, Bulgaria; BG_C, Central Bulgaria; TR, Turkey; MK, North Macedonia; RS_E, Eastern Serbia; RS_C, Central Serbia. *Rhinusa dieckmanni* host plant associations with different *Linaria* spp. are designated according to color of squares as shown in the figure legend. Range of seed capsules size per each *Linaria* host plant of *R. dieckmanni* is presented between solid and dashed lines in its corresponding color. Data on the range of seed capsule size were obtained from Sutton (1988).

distributed haplotype in Europe was also recorded in North America, as well as haplotype H20, which was recorded in the native range from all three toadflax species included in this study. Haplotype H34 was not detected in the native range or in the North Macedonian populations; however, this haplotype is closely related to haplotypes associated with *L. dalmatica* ssp. *macedonica*, thus supporting their conspecific nature. Thus, according to combined results of morphological and molecular studies, all entities sampled from three toadflax species at 37 locations across southeastern Europe and Turkey and including "Dalmatian host race" specimens collected in North America, belong to a single taxon, *Rhinusa dieckmanni*. In addition, the recorded genetic distance between *R. dieckmanni* and *R. antirrhini* s.str. associated with *L. vulgaris* is 13.1% (accession number ON934911) (Fig. 6), confirming differences at species level (Brower, 1994).

Results obtained by additional sequencing of the mitochondrial *16S* gene positioned opposite to the *COII* gene within the circular structure of mtDNA and, nuclear *EF-1α* gene confirm that all analyzed geographically distant and host associated populations collected from *L. genistifolia*, *L. dalmatica*, *L. dalmatica* ssp. *macedonica*, and *L. grandiflora* are conspecific. Analysis revealed less expressed genetic diversity due to the more conservative and slow evolving nature of these genes. A total of seven *16S* haplotypes were recorded across 44 sequenced specimens and seven *EF-1α* haplotypes from 47 specimens. The genealogy using a statistical parsimony network of the recorded *16S* (Fig. 6b) and *Ef-1α* (Fig. 6c) haplotypes follows results obtained by analysis of *COII* gene and undoubtedly shows the common species origin in all specimens associated with *L. genistifolia*, *L. dalmatica*, and *L. grandiflora*.

The AMOVA results for the grouping according to affiliations with geographical region showed that 16.65% of mt*COII* genetic variation is explained by variation among these groups, whereas most of the variation in the total molecular variance (72.5%) is explained by variation within the populations. A similar but more pronounced pattern was observed when populations are grouped according to host plant: 5.67% for the variation among groups and 80.96% for the intra-population variation. Accordingly, F_{CT} values were low and non-significant. For both groupings, F_{ST} values were low, however significant, at 0.275 and 0.19, respectively (Table 1).

3.4. Redescription of *Rhinusa dieckmanni*

Morphological analysis combined with genetic and biological data on the weevils associated with three closely related toadflax species, *L. dalmatica*, *L. genistifolia* and *L. grandiflora*, revealed the need for a redescription of *R. dieckmanni*, a taxon previously considered to be a highly endemic weevil species known only from Bulgaria. This redescription is additionally relevant because this species, targeted as a classical biological control agent for Dalmatian toadflax under the name *R. antirrhini* "Dalmatian host race," is confirmed as established as the result of its 1990s introduction to North America.

Rhinusa dieckmanni (Behne, 1988).

Gymnetron (*Rhinusa*) *dieckmanni* Behne, 1988: 31. Type locality: Bulgaria, Mt. Rila, Rila Monastery environment, 1200 m.

Rhinusa dieckmanni (Behne). Caldara et al., 2010: 52.

Diagnostic redescription. Body black, oval, stout. Body length 1.6–4.1 mm in males and 1.6–4.4 mm in females (rostrum excluded). Rostrum shorter in males (rostrum length/pronotum length 0.70–1.05), distinctly longer in females (rostrum length/pronotum length 0.90–1.40); rostrum in lateral view almost straight, abruptly narrowed and slightly upward in apical part; in dorsal view distinctly narrower in distal part, with well

Fig. 5. Variability in floral size (above) and seed capsules size (below) among populations of *Linaria* species and subspecies utilized for development by *Rhinusa dieckmanni*: a) *L. grandiflora* (Kirşehir, Turkey); b) *L. dalmatica* (Mount Rila, Bulgaria, type locality of *R. dieckmanni*); c) *L. dalmatica* (Pirot, Eastern Serbia); d) *L. dalmatica* ssp. *macedonica* (Prilep, North Macedonia); e) *L. genistifolia* (Bansko, southeastern Bulgaria).

visible scrobes, pronotum conical, with dense and regular punctures, covered with recumbent to subrecumbent yellowish-white scales. Elytra subrectangular, with subrecumbent to suberect yellowish-white scales arranged in one-two irregular rows on each interstria, denser along apical part of interstria. Body of penis elongated, with concave sides, narrowest at middle then enlarged to near apex.

Variability. Rhinusa dieckmanni is highly variable in size, which is a consequence of the seed capsule size of the respective host utilized for larval development. Body length (rostrum excluded) ranges from 1.6 to 4.4 mm, with rostrum length ranging from 0.2 to 1.4 mm.

Comparative notes. Rhinusa dieckmanni belongs to the monophyletic *R. antirrhini* species group (Caldara et al., 2010), which is represented in the Balkans and in Turkey by three species: *R. antirrhini*, *R. florum* and *R. exigua* Caldara & Korotyaev. The smallest specimens of *R. dieckmanni* are more closely related to *R. antirrhini*, from which they differ by a longer and more tapered rostrum in its apical part, especially in females, and by a more rounded and more transverse pronotum (Behne, 1988).

Bionomics. All the species of the *R. antirrhini* group appear to be monophagous (Hernández-Vera et al., 2010), whereas the host plants utilized for larval development of *R. dieckmanni* are equally *L. dalmatica*, *L. genistifolia* and *L. grandiflora*. This was confirmed on several locations in Eastern Serbia and North Macedonia where *L. dalmatica* and *L. genistifolia* grow in syntopy. The typical *L. grandiflora* (following Sutton, 1988) was recorded only in the Anatolian area of Turkey, where *R. dieckmanni* utilizes only this plant for larval development, while other closely related toadflax species considered as subspecies of *L. genistifolia* in Middle Asia are utilized by different seed-feeding *Rhinusa* species (Hernández-Vera et al., 2010).

Rhinusa dieckmanni is univoltine and usually found in lowlands, hilly slopes, and mountain meadows at elevations up to 2000 m, following their host plants. The adults overwinter in soil and litter proximate to host plants, emerging in early June. Copulation occurs shortly after, with the egg-laying period lasting from the end of June until the beginning of October. Oviposition occurs on the widest part of the developing ovary. Prior to oviposition, the female makes a hole with its rostrum through the sepal leaves to penetrate the ovary. During oviposition, females secrete a fluid that fixes the egg to the ovule. Females primarily lay one egg per ovary. Egg deposition triggers a tissue reaction manifested as a semi-gall formation at the wound site. Ovule tissue around the egg experiences proliferation followed by cell hypertrophy which serves as the initial food source for newly hatched larvae.

Rhinusa dieckmanni is very common on its host plants in southeastern Europe and Middle Asia. This species co-occurs with *R. neta*, an oligophagous seed feeding weevil which develops on various toadflax plant species, and with *R. florum*, which induces galls on seed capsules and is associated with *L. genistifolia* and *L. dalmatica*. Even though these two species inhabit the same niche for larval development as *R. dieckmanni*, competition between them has not been observed. During the 1990 s, *R. dieckmanni* was introduced to northwestern North America under the name of *R. antirrhini* “Dalmatian host race”.

4. Discussion

To obtain sufficient data for the present study, a total of 180 weevils associated with 37 plant populations in southeastern Europe and Turkey were evaluated, including the *R. dieckmanni* population from the type locality. Within their native range, these weevils are phenotypically variable, but consistently (including in syntopy) associated with only three toadflaxes, *L. genistifolia*, *L. dalmatica* and *L. grandiflora*. Genetic studies confirmed that weevils from all studied plant populations belong to the unique taxon *R. dieckmanni*, first described from Mount Rila in southwestern Bulgaria. Furthermore, results of the analysis of molecular variance are in accordance with the results of the statistical parsimony analysis and resulting haplotype network.

The taxonomic status of the *R. dieckmanni* host plants remains blurred, ranging from a very broad species concept where *L. dalmatica*

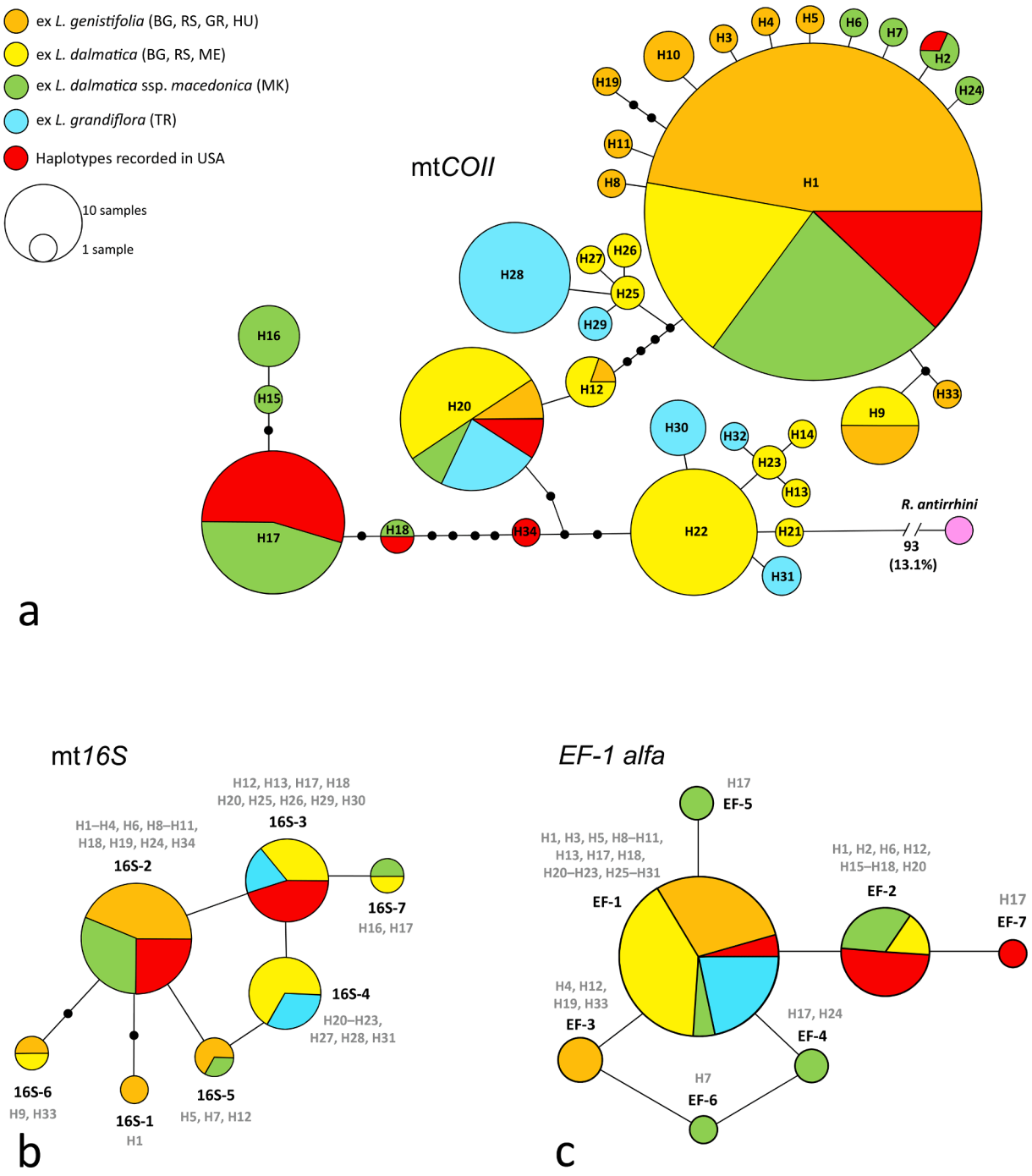

Fig. 6. Statistical parsimony networks of *Rhinusa dieckmanni* haplotypes inferred from (a) mitochondrial cytochrome oxidase subunit II (*COII*) gene, (b) mitochondrial *16S* ribosomal RNA gene, and (c) nuclear elongation factor 1-alfa (*EF-1α*) gene sequences. The *COII* gene analyzes included *R. dieckmanni* specimens from 37 locations in southeastern Europe and Turkey associated with *L. dalmatica*, *L. dalmatica* ssp. *macedonica, L. genistifolia* and *L. grandiflora,* along with 24 specimens originating from North America. The *16S* and *EF-1α* gene analyzes included subset of specimens representing diverse *COII* gene haplotypes. Per each gene haplotypes are denoted in black font: H1 to H34 for *COII*, 16S-1 to 16S-7 for *16S*, and EF-1 to EF-7 for *EF-1α*. Next to the *16S* and *EF-1α* gene haplotypes are their associated *COII* haplotypes denoted in grey font. Circle sizes are proportional to haplotype frequency. Black dots represent missing haplotypes. Number above the solid broken line represents genetic distance from *R. antirrhini* specimens associated with *L. vulgaris*. Geographic origin of sampled *R. dieckmanni* populations is designated as follows: BG, Bulgaria; RS, Serbia; GR, Greece; HU, Hungary; ME, Montenegro; MK, North Macedonia; TR, Turkey; and USA, United States of America (all ex *L. dalmatica*).

and *L. grandiflora* are conspecific with *L. genistifolia* (Chater et al., 1972), or variable stabilized hybrids (Davis, 1978), to separate taxa (Sutton, 1988). Despite the extraordinary phenotypic plasticity of these toadflax species as observed in numerous plant populations across their collective distribution range at both the intra- and inter-population levels, for practical reasons, this study adopted Sutton's (1988) toadflax nomenclature. The phenotypic plasticity of these toadflaxes parallels the phenotypic plasticity observed in *R. dieckmanni* adults. Thus, considering known host plant usage, *R. dieckmanni* in practice recognizes these three toadflax species as effectively a single entity acceptable for egg deposition and larval development. Similarly, in the native range, at least two other weevil species from the tribe Mecinini, e.g., *Rhinusa rara* Toševski & Caldara and *Mecinus janthiniformis*, indiscriminately use both *L. genistifolia* and *L. dalmatica* for oviposition and larval development

Table 1
Hierarchical analysis of molecular variance (AMOVA) based on mt*COII* data of *R. dieckmanni* populations.

Source of variation	df	Variation (%)	Interpopulation fixation indices	
			F_{CT}	F_{ST}
Geography				
Among groups	3	16.65	0.1665 ^{ns}	0.275*
Within groups	4	10.88		
Within populations	136	72.5		
Host plant				
Among groups	2	5.67	0.0567 ^{ns}	0.19*
Within groups	1	13.37		
Within populations	176	80.96		

df – degrees of freedom, ns – not significant, * $P < 0.001$.

(Toševski et al., 2011; 2015), which strongly supports the conspecific relationship of these two host plant species.

For a long time, the seed-feeding weevils associated with *L. genistifolia* and *L. dalmatica* were misidentified given their morphologically cryptic nature within the *R. antirrhini* complex (Hernández-Vera et al., 2010). During the 1990 s, the "Dalmatian host race" was introduced to North America under the name *R. antirrhini*, but in contrast to the widespread distribution of this species (s. str.) across *L. vulgaris* populations in North America, the "Dalmatian host race" experienced conspicuous adversity in establishing significant populations in the adopted range, with negligible landscape-level impact on *L. dalmatica* (Sing et al., 2016). Difficulties with establishment were especially common in high elevation habitats with shorter summers and colder winters, although the release of such agents in this type of habitat was of primary interest (Turner, 2008). From the beginning of the biological control program for *Linaria* spp., agent populations were selected for release based on their adaptation to the colder climate in northwestern North America. For this reason, the population associated with *L. dalmatica* ssp. *macedonica* was supposedly the best choice, i.e., originating from the appropriate plant species and high elevations (about 1000 m), and thereby tolerant of cold winters (McCartney et al., 2019).

The widespread occurrence of European and Middle Asian populations of *R. dieckmanni* in their native range was confirmed in habitats ranging from the seashore with very hot summers and mild winters (e.g., Asprovalta, Greece), to habitats at high elevation (e.g., Anatolian Plateau, Turkey; meadows on Mount Rila, Bulgaria above 1900 m). Mount Rila's environment is characterized by short summers and extreme cold temperatures during winter, combined with heavy snowfall. Lack of climatic adaptation is therefore likely not the reason for the low establishment success and abundance of the "Dalmatian host race" after release.

Rhinusa dieckmanni specimens from Turkey and Mount Rila populations were larger and had a longer rostrum compared to the phenotype originating from *L. dalmatica* ssp. *macedonica* and used for the North America introductions. Our in-field observations suggest that the long rostrum phenotype was correlated with plant phenotypes dominated by large flowers, strongly developed and pulpous petals, and large seed capsules. This plant phenotype is also typical for invasive Dalmatian toadflax populations in North America. In contrast, the petals of *L. dalmatica* ssp. *macedonica* and other surveyed *L. dalmatica* and *L. genistifolia* were relatively thin and yielding, with variable seed capsule sizes.

This is in accordance with the observed variation in *Linaria* seed capsule size reported by Sutton (1988), ranging for *L. dalmatica*, *L. grandiflora,* and *L. genistifolia* between 4 and 7 × 4.5–6, 8–11 × 8–10, and 2.5–6 × 2.5–6.5 (mm), respectively (Fig. 4). Nevertheless, our toadflax species and subspecies variation in seed capsule size was found to be even higher (Fig. 5; data not shown). Although more research is needed to better understand the relationship between seed capsule size and rostrum length, it is important to note that flower and seed capsule size vary on plants throughout the flowering season, which appears to influence differences in weevil rostrum and body size *in situ*. Due to the oviposition requirements of this species, rostrum length is an important factor that enables females to successfully deposit their eggs inside the ovary, thereby ensuring normal larval development. Thus, the females' ability to reach the ovary during oviposition is directly correlated with phenotypic characters of the petals, such as size and thickness, which in turn influence rostrum length within *R. dieckmanni* populations. This is in accordance with general adaptive patterns often recorded in phytophagous insects with regard to variation in host plant morphology (Bernays, 1991; Carroll and Boyd, 1992). In addition, one of the main conclusions of our study is that there is no genetic difference among weevils inhabiting *L. dalmatica*, *L. genistifolia* and *L. grandiflora*. This is based primarily on the mitochondrial *COII* gene sequencing supported by the nuclear *EF-1α* gene sequencing. Although there is no genetic structuring associated with host-plant use of the weevils based on mt*COII*, this does not completely rule out host-specific cryptic segregation. Future studies should focus on determining whether weevils have recently adapted to new host plants using faster-evolving markers (such as microsatellites or genome-wide SNPs).

The results presented in this paper are an example of how an in-depth assessment of biological control agents can be conducted retrospectively, using an integrative approach combining morphological, ecological, and molecular techniques to reveal the species status of an introduced biocontrol agent for which the taxonomical position has been unclear or misinterpreted for a long time. During the 1990 s, the taxonomic status of introduced biological control agents was traditionally determined by taxonomic specialists following the typological species concept. Following pre-release host specificity studies (Groppe, 1992), all putative *R. antirrhini* destined for field release in North America were defined as "Dalmatian host race" and collected exclusively from *L. dalmatica* ssp. *macedonica* for introduction.

A new taxon, *R. dieckmanni*, intentionally introduced as *R. antirrhini* "Dalmatian host race" in the 1990s, has been confirmed in association with invasive Dalmatian toadflax in North America. The mitochondrial haplotypes recorded in association with North American populations of *L. dalmatica* confirm North Macedonia as the country of origin for *R. dieckmanni*, now established in northwestern USA. Observed variability in rostrum length, i.e., phenotypic plasticity within weevil populations, implies that morphological variability in *R. dieckmanni* is not a consequence of phylogenetic constraints but rather an adaptation to variable host plant specific phenotypes. The origins and mechanism of the observed phenotypic plasticity exceed the focus of this paper. However, the observation that phenotypic changes in rostrum size are adaptive and a result of plasticity in host plant seed capsule size is supported by the presented results. Therefore, the question arising nearly thirty years after its first introduction is, "Has the appropriate phenotype of *R. dieckmanni* been introduced into North America?" With the benefit of additional knowledge, the answer must be "no"! A more suitable choice would have been to introduce individuals from populations with a longer rostrum. Considering the phenotypic plasticity of the host plants used by *R. dieckmanni*, processes of natural selection should be expected to act on established weevil populations to adequately respond, in terms of rostrum size, to the selection pressures imposed by predominant host plant phenotypes (Carroll and Boyd, 1992). In this case, the adaptive plasticity of rostrum size in *R. dieckmanni* will be influenced by the ecological properties of available host plants, which in time may increase the abundance of the appropriate phenotype of this weevil in North America. The North American populations of Dalmatian toadflax are most phenotypically similar to L. dalmatica and L. grandiflora populations from Mount Rila (Bulgaria), Anatolia (Turkey), and Staničenje (Eastern Serbia), respectively. Thus, additional introductions of the long rostrum phenotype across R. dieckmanni populations seems to be the most appropriate tactic to accelerate the widespread establishment of this weevil for the control of invasive North American Dalmatian toadflax.

CRediT authorship contribution statement

Ivo Toševski: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Sharlene E. Sing:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – review & editing. **Roberto Caldara:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **David K. Weaver:** Investigation, Writing – review & editing, Project administration, Funding acquisition, Supervision. **Jelena Jović:** Formal analysis, Investigation, Validation, Visualization, Writing – review & editing. **Oliver Krstić:** Formal analysis, Investigation, Data curation, Writing – review & editing. **Hariet L. Hinz:** Investigation, Writing – review & editing, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

DNA sequences are available in the GenBank database, accession numbers are listed in the Results and in Appendix A. Tables S1 and S2. All other relevant data are within the paper and its Appendix A.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2023.105270.

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