**Host associated genetic divergence and taxonomy in the *Rhinusa pilosa*
Gyllenhal species complex: an integrative approach**

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Running title: Taxonomy of the *Rhinusa pilosa* complex

37 **Abstract**

38

39 A combined taxonomic, morphological, molecular and biological study revealed that stem galling
40 weevils from the genus *Rhinusa* associated with toadflaxes from the genus *Linaria* (Plantaginaceae)
41 are composed of three different species: *Rhinusa pilosa*, *R. brondelii* and *Rhinusa rara* **sp.n.** The
42 authentic field host plants are respectively, *Linaria vulgaris*, *L. purpurea* and *L. genistifolia* /*L.*
43 *dalmatica*. These weevil species can be distinguished from each other by a few subtle
44 morphological characteristics, mainly in the shape of the rostrum and of the integument. An analysis
45 of the mitochondrial (*COII* and *16S*) and nuclear (*EF-1 α*) sequence data revealed high genetic
46 divergence among these species. Uncorrected pairwise distances on mt*COII* gene were 14.3%
47 between *R. pilosa* and *R. brondelii*, 15.7% between *R. pilosa* and *R. rara*, while *R. brondelii* and *R.*
48 *rara* are approximately 11% divergent from each other. Divergences obtained on *16S* and nuclear
49 *EF-1 α* gene was congruent. However, substantial intraspecific mitochondrial divergence was
50 recorded for all studied populations of *R. pilosa* s.s. showing two mtDNA lineages, with estimated
51 *COII* and *16S* divergences of 4% and 1.6%, respectively. Nuclear pseudogenes (Numts) and
52 *Wolbachia* influence, although recorded within both lineages, were excluded as possible causatives
53 of the mtDNA divergence, while *EF-1 α* indicated absence of lineage sorting. Species from the *R.*
54 *pilosa* complex are estimated to have diverged from each other approximately 7.2 Ma (late
55 Miocene), while *R. brondelii* and *R. rara* have diverged from each other about 4.7 Ma (early
56 Pliocene).

57

58 **Introduction**

59

60 The weevil genus *Rhinusa* Stephens (Curculionidae, Mecynini) is composed of approximately 40
61 species, all with a Palearctic distribution (Reitter, 1907; Caldara, 2001; Caldara, 2013). Based on

62 morphological characters all species from the genus *Rhinusa* have been divided into ten species
63 groups (Caldara *et al.*, 2010). A phylogenetic analysis based on morphology suggests that the genus
64 *Rhinusa* is monophyletic and sister to the Palaearctic species of the genus *Gymnetron* Schoenherr,
65 1825 (Caldara 2001). However, a recent comprehensive molecular phylogenetic analysis does not
66 support monophyly for either genus, but does suggest an origin for all taxa from an ancestral
67 *Gymnetron* species from southern Africa. It was concluded that subsequent range expansion from
68 southern Africa was followed by extensive species radiation in the western Palaearctic, with the
69 earliest divergence estimated to date back to circa 27 million years ago (mya) (Hernández-Vera *et*
70 *al.*, 2013).

71
72 All known species in the genus *Rhinusa* use species of Plantaginaceae (*Linaria*, *Antirrhinum*,
73 *Mesopates*, *Chaenorhinum* and *Kickxia*) and Scrophulariaceae (*Verbascum*, *Scrophularia*) as host
74 plants, while *Gymnetron* from the Palaearctic region are only associated with species from the genus
75 *Veronica* (Plantaginaceae). Larvae of both genera are endoparasitic, with most species being seed
76 feeders, with several species utilizing stems for larval development and only a few being gall
77 inducers. Within *Rhinusa*, two closely related species, *Rhinusa pilosa* (Gyllenhal, 1838) and *R.*
78 *brondelii* (Brisout de Barneville, 1862), form stem galls in toadflaxes (Caldara *et al.*, 2008; Caldara
79 *et al.*, 2010). The taxonomic position of these two species was recently clarified by Caldara *et al.*
80 (2008) by the designation of the lectotype of *Gymnetron pilosum* Gyllenhal and the neotype of
81 *Gymnetron pilosum brondelii* Brisout de Barneville. The authors concluded that *R. pilosa* and *R.*
82 *brondelii* can be distinguished from each other by biological, genetic and subtle morphological
83 differences. *Rhinusa pilosa* was defined as associated with *Linaria vulgaris* (L.) Mill., while for *R.*
84 *brondelii* a broader host range was described which included *L. purpurea* (L.) Mill. (Italy), *L.*
85 *genistifolia* (L.) Mill. and *L. dalmatica* (Serbia), *L. reflexa* (L.) Desf. (Tunisia) and *L. gharbensis*
86 Bat. & Pit. (Morocco).

87

88 Several of the host species associated with *R. pilosa* and *R. brondelii* are invasive in North America
89 and in 1987 a biological control program was initiated for *Linaria vulgaris* and *L. dalmatica*. It was
90 considered that gall-inducing weevils associated with toadflaxes could be preferable bio-control
91 candidates since gall makers typically have a narrow host range and can negatively affect host
92 plants (Manni, 1964; Harris & Shorthouse, 1996). The intentional use of beneficial organisms in
93 classical biological control programs places species delimitation at the center of concern for
94 practitioners, and the determination of species limits within a potential biological control agent is
95 essential for the assessment of its biological properties and host specificity (Schaffner, 2001). This
96 applies particularly to cryptic species that are, by definition, difficult to distinguish based on
97 phenotypic characters (Bickford *et al.*, 2007).

98
99 The recent wide use of molecular techniques substantially improves possibilities for reliable
100 taxonomic clarification in problematic taxa, delineation of cryptic species, estimation of population
101 structure and genetic variability (Krosch *et al.*, 2013; Tay *et al.*, 2012), and fast species
102 identification, particularly in applied studies (Gaskin *et al.*, 2012). Most frequently, the target
103 genetic marker of choice in such studies is mitochondrial. Although broadly exploited, particularly
104 after the barcode initiative (Hebert *et al.*, 2003), analyses based only on mitochondrial markers may
105 lead to erroneous results because of relatively frequent unintended co-amplification of nuclear
106 pseudogenes (numts). This is usually of concern when deep mitochondrial divergence is observed
107 within populations that are considered as unique taxon (Bensasson *et al.*, 2001; Song *et al.*, 2008;
108 Kindler *et al.*, 2012). Recently, more comprehensive phylogenetic studies have shown that deeper
109 mitochondrial divergence within populations of particular arthropod species can be driven by
110 presence of parasitic *Wolbachia* and its ability to manipulate with mitochondrial DNA of the host
111 (Werren *et al.*, 2008; Smith *et al.*, 2012). Due to these potential complications, precautionary
112 measures and a holistic approach should be undertaken when choosing appropriate markers and
113 methods of phylogenetic analyses for cryptic species determination and evaluation.

114

115 Over the last ten years the potential of stem gall forming weevils as potential biological agents for
116 the control of invasive toadflaxes has been the subject of several studies (e.g. Toševski *et al.*, 2005).
117 Host suitability studies of the stem galling weevils from the *R. pilosa* species group (Toševski *et al.*,
118 2012) have suggested highly host-specific interactions between weevil populations and their
119 respective toadflax host, with elements of cryptic speciation, previously suggested by Caldara *et al.*
120 (2008). These findings reinforce the need to evaluate the taxonomic placement of populations of
121 *Rhinusa* associated with stem gall induction in other toadflax species in the western Palaearctic.
122 This is of particular importance, as the consequences will underpin decisions regarding these taxa as
123 potential bio-control agents. Here we present the results of morphological, biological and genetic
124 studies on stem gall inducing weevils within the *R. pilosa* species group sampled from toadflaxes in
125 Europe to better understand their taxonomic status, nomenclatural positions and the approximate
126 temporal framework for their evolution and historical biogeography.

127

128 **Materials and Methods**

129

130 *Acronyms.* The collections housing material studied in this revision are abbreviated as follows (with
131 their curators in parentheses):

132

133 CABIC - CABI collection, Delemont, Switzerland
134 CBCM - Cosimo Baviera collection, Messina, Italy.
135 DEIM - Deutsches Entomologisches Institut, Müncheberg, Germany (L. Behne)
136 GOCA - Giuseppe Osella collection, L'Aquila, Italy
137 ITCB - Ivo Toševski collection, Belgrade, Serbia.
138 JFCH - Jan Fremuth collection, Hradek Králové, Czech Republic
139 LMCP - Luigi Magnano collection, Poggibonsi, Italy
140 MNHN - Muséum National d'Histoire Naturelle, Paris, France (H. Perrin).
141 MSNG - Museo civico di Storia Naturale, Genova, Italy (R. Poggi)
142 MSNM - Museo civico di Storia Naturale, Milano, Italy (C. Pesarini, F. Rigato)
143 NHRS - Naturhistoriska Riksmuseet, Stockholm, Sweden (B. Viklund).
144 RCCM - Roberto Caldara collection, Milano, Italy.
145 SMTD - Staatliches Museum für Tierkunde, Dresden, Germany (O. Jäger, K. Klass)

146

147 *Specimens examined.* This study is based on a previous examination of the type specimen series and
148 archived specimens published by Caldara *et al.* (2008), and newly collected material for
149 morphological and genetic analysis (Appendix S1). Several hundred specimens were examined in
150 order to determine the geographical distribution of the studied taxa. Specimens for molecular
151 analysis sampled from *Linaria vulgaris*, *L. genistifolia* and *L. purpurea* were reared from galled
152 plants collected in Hungary (*L. vulgaris*, *L. genistifolia*), Romania (*L. vulgaris*), Serbia (*L. vulgaris*,
153 *L. genistifolia*, *L. dalmatica*), South Russia (*L. genistifolia*), and South Italy (*L. purpurea*) (Fig. 1;
154 Appendix S1). In addition, the neotype of *Gymnetron pilosum* var. *brondelii* Brisout de Barneville,
155 1862 (des. Caldara) and 14 archival voucher specimens deposited in the MNHN and previously
156 ascribed to *R. brondelii* by Caldara *et al.* (2008) were submitted for DNA extraction and sequencing
157 to reassess their taxonomic status (Appendix S2).

158

159 *Morphological study.* In accordance with Caldara *et al.* (2010), we considered the following as
160 possible diagnostic characters: body length (rostrum excluded), shape of head, eye, antennae,
161 rostrum, prothorax, elytra and abdomen; colour, sculpture and vestiture of rostrum, pronotum and
162 elytra and shape of penis. In the following treatment of species, due to the great similarity of many
163 characters, we report an accurate diagnosis instead of a complete description, in order to avoid
164 describing character variation that does not differ among the species of the *R. pilosa* group.

165

166 *Gall inducing and host suitability study.* Gall induction studies were carried out with weevil
167 populations associated with *L. vulgaris* (= *R. pilosa*) and population of weevils associated with *L.*
168 *genistifolia* and *L. dalmatica* as a part of host specificity tests performed with both species within
169 the framework of their assessment within biological control studies (Toševski *et al.*, 2012). In two
170 trials, post hibernated females sampled from *L. vulgaris* and *L. genistifolia* were set up for
171 oviposition on their respective field host plants (control series) and the field host plant of the other

172 population (test series). In accordance with their biology and phenology, the no-choice gall
173 induction test was set up at the beginning of April when oviposition begins on both host species
174 (Toševski *et al.*, 2005). During testing, females were transferred to fresh potted plants every 3-4
175 days for oviposition. Each plant was checked for oviposition and gall induction, while the number
176 of oviposition marks per induced gall was also recorded. Galls were dissected for adult development
177 during the first week of July. The number of induced galls, total number of ovipositions, and
178 successful development to adult were recorded from each plant. Significance of obtained results of
179 the larval development experiment were assessed by total and mean development rate on control
180 and test plants and the percent of recorded ovipositions that resulted in development to adult.

181

182 *Material for DNA study.* Even though *R. pilosa* is inferred to have a broad geographic range, the
183 species is known from only a few localities, suggesting extremely rare and local occurrence. To
184 date, all confirmed localities for *R. pilosa*, based on material in public and private collections, are
185 located between latitudes 43°N and 59°N within the Western Palearctic (Fig. 1). Several surveys
186 performed across central and western Europe were unsuccessful in an attempt to collect *de novo*
187 material for genetic analysis. *Rhinusa pilosa* stem galls on its host plant species *L. vulgaris* have
188 been found only in Serbia (12 populations), Hungary (1 population) and in Romania (1 population).
189 Stem galls on other toadflax species were recorded for *L. genistifolia* and *L. dalmatica* in Serbia (3
190 populations), *L. genistifolia* in Hungary (1 population) and *L. genistifolia* in southern Russia (1
191 population). In addition, stem galls were found on *Linaria purpurea* (L.) Mill. within high mountain
192 meadows on Mt. Etna (Sicily) and Montalto peak (Aspamonte massif, Calabria). All weevils used in
193 this study were reared from field collected galls, and stored in 96% ethanol at -20°C until DNA
194 extraction immediately after being collected. Weevils collected from a single gall were stored
195 separately in 2 ml cryovials (Sarstedt, Germany), and it was considered that a given gall induction
196 event was the product of a single female. Thus, to obtain a representative overview of genetic
197 diversity of the studied populations, only one adult per sampled gall was submitted for molecular

198 analyses. After DNA extraction, weevil specimens were prepared as voucher dry specimens for
199 taxonomic study.

200

201 *DNA extraction.* Individual weevils were punctured at the second thoracic sternite and total DNA
202 was extracted using the QIAGEN Dneasy® Blood & Tissue Kit (Germany), according to
203 manufacturer's instructions. The same procedure was used to extract DNA from dry neotype
204 specimen of *Rhinusa brondelii* and other 14 specimens of *Rhinusa brondelii* originated from North
205 Africa, all deposited at MNHN collection in Paris.

206

207 *PCR amplification and sequencing.* The mitochondrial cytochrome oxidase subunit II gene (*COII*)
208 was amplified using the primers TL2-J-3038 (Emerson *et al.*, 2000) and TK-N 3782 (Harrison
209 Laboratory, Cornell University, Ithaca, NY, USA) (Table 1). Amplification reactions were
210 performed in a 20- μ l final reaction volume containing Kapabiosystems high yield reaction buffer A
211 with 1.5 mM MgCl₂ (1x), an additional 3.5 mM MgCl₂, 0.8 mM of each dNTP, 0.75 μ M of each
212 primer, 0.75 U of KAPATaq DNA polymerase (Kapa Biosystems, Inc., Woburn, MA, USA) and 1
213 μ l of DNA extract. PCRs were carried out in a Mastercycler ep gradient S (Eppendorf, Germany)
214 applying the following thermal steps: 95°C for 5 min (initial denaturation), 40 cycles at 95°C for 1
215 min, 1 min at 45°C (annealing), 72°C for 2 min, and a final extension at 72°C for 10 min. The
216 mitochondrial *16S* ribosomal RNA gene (*16S*) was amplified using the primers 16Scf1 and 16Sbr1
217 (Table 1). Reactions were performed with 1 x High Yield Reaction Buffer A with Mg, 2.25 mM
218 MgCl₂, 0.6 mM of each dNTP, 0.5 μ M of each primer, 1U of KAPATaq polymerase and 1 μ L of
219 DNA template in a 20- μ L final volume. PCRs were carried out using the following thermal profile:
220 94°C for 1 min 30 s, 35 cycles at 94 °C for 45 s, 50°C for 1 min, 72°C for 1 min 30 s, and a final
221 extension at 72°C for 7 min. Amplifications of the nuclear gene elongation factor-1 α (*EF-1 α*), were
222 performed with the primers EF1-Bf and EF-Br (Hernandez-Vera *et al.*, 2013; Table 1). Reactions
223 were performed in a 20- μ l final reaction volume under the same reaction and thermal conditions as

for *I6S* amplification, except for the number of amplification cycles which was raised to 40. PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN), and sequenced by BMR Service (Padova, Italy) and Macrogen (Seoul, Korea). For most specimens, sequences of the complete *COII* gene were obtained with the forward primer only, whereas if needed, sequencing was performed with both primers to read sequences of full-length PCR products. Sequences of the *I6S* were obtained with the reverse primer only. All specimens were sequenced in both directions for the *EF-1 α* amplicon. Sequences are available from GenBank under accession numbers KJ420489-KJ420509 for the *COII* gene (Appendix S1), KJ420513-KJ420523 for the *I6S* ribosomal RNA gene (Appendix S3) and KJ620000- KJ620004 for the *EF-1 α* gene (Appendix S5).

Evolutionary tree construction and estimation of divergence times. Sequences were edited with FinchTV v.1.4.0 (www.geospiza.com), and aligned with ClustalW within the MEGA 5 software (Tamura *et al.*, 2011). Aligned sequences were truncated to 670 bp from the 3' end for phylogenetic analyses. Maximum parsimony and neighbor-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). Five hundred bootstrap replicates were performed to assess branch support of the resulting tree topologies. Bayesian analyses were carried out with the program MRBAYES version 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Two simultaneous runs were executed 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 that best fits the data with priors set to the default values. Posterior probabilities were assessed with Tracer 1.5.0 (<http://beast.bio.ed.ac.uk>) to ensure that sampling had reached stationarity within the burn-in. The tree was rooted using *Rhinusa melas* (Boheman, 1838) as an outgroup (GenBank accession number KJ420488).

250

251 In the absence of geological and/or fossil calibration points related with weevils from the genus
252 *Rhinusa*, we have taken a Bayesian approach to estimate the age of the most recent common
253 ancestor (mrca) of the whole group and the inferred lineages. Mitochondrial rates have been
254 proposed for arthropods in the range of 1.2-4.9% pairwise divergence per million years (myr)
255 (DeSalle *et al.*, 1987; Brower, 1994; Caccone & Sbordoni, 2001). To take this reported variance
256 into account we applied per lineage rates of 0.006 and 0.0245 substitutions per site per myr
257 (subs/s/myr) which correspond to 1.2% and 4.9% pairwise divergence per myr, respectively.
258 Furthermore, we applied a mean lineage rate of 0.0152 subs/s/myr which is equivalent to 3.05%
259 pairwise divergence per myr, following Cicconardi *et al.* (2010), who demonstrated the mean
260 substitution rate for the COII gene within Coleoptera to approximate the rate across the
261 Pancrustacea. The mean lineage rate of 0.0152 subs/s/myr applied in our analysis closely matches
262 the rate of 0.0134 subs/site/myr proposed by Pons *et al.* (2010), and rate of 0.0177 subs/site/myr
263 proposed by Papadopoulou *et al.* (2010). In order to estimate divergence times among genetic
264 lineages within the *R. pilosa* species group, we used BEAST version 1.7.4 (Drummond *et al.*, 2012).
265 For our analyses we applied a random local clock model (Drummond & Suchard, 2010) with the
266 average number of substitutions per site across the tree averaged to be 1.525% per million years.
267 Initial substitution model parameter values were Hasegawa-Kishino-Yano model with gamma
268 distribution (HKY+G), selected according to the results of jMODELTEST, with unconstrained prior
269 distributions. A separate demographic model of constant population size was applied, in the form of
270 a coalescent prior, to clades conforming to genetic variation within host associated lineages, and a
271 Yule tree prior was used for the basal branches connecting these. Input files were generated with
272 BEAUTI version 1.7.4 (Drummond *et al.*, 2012). Two runs were conducted for the lowest, highest
273 and mean substitution rates, each consisting of 30 000 000 generations, and sampling every 1000
274 generations. For each of the three rate values the two runs were combined after checking sampling,
275 mixing and convergence of Markov chains to a stationary distribution. Estimated lineage divergence

times were summarized and expressed as mean values in million years (myrMa) using Tracer 1.5.0 with lower and upper bounds of the 95% highest posterior density intervals (HPD).

Haplotype network construction. Relationships resulting from intrinsic population-level processes (e.g. persistence of ancestral haplotypes, multifurcations, recombination and horizontal transfer) are better visualized in reticulated graphs or networks (Posada & Crandall, 2001). Gene genealogies of *COII* and *EF-1 α* were inferred using two approaches for haplotype network construction. Median-joining networks (Bandelt *et al.* 1999) were calculated with the program NETWORK version 4.612 (www.fluxus-engineering.com) keeping the parameter $\epsilon = 0$, and the post processing option for maximum parsimony calculation. This method starts with minimum spanning trees combined within a single network and then, to reduce tree length, median vectors (consensus sequences) are added. Such vectors can be interpreted as possibly extant unsampled sequences or extinct ancestral sequences (Bandelt *et al.* 1999). In addition, TCS version 1.21 (Clement *et al.* 2000) was employed to infer haplotype networks using statistical parsimony (Templeton *et al.* 1992) with a confidence limit of 95%.

Molecular identification of archival museum specimens. Considering that mitochondrial DNA (mtDNA) in dry insect material is frequently highly degraded and fragmented, for the analysis of archival voucher specimens, a set of three primer pairs 248Ft/Rt, 450Ft/Rt and 515Ft/Rt were designed for the amplification of short phylogenetically informative amplicons (SPIAs, Hernández-Vera *et al.*, 2013) of the *COII* gene, comprising 85, 88 and 131 bp in length (Table 2, Fig. 2). Primers were positioned within the *COII* gene sequence for the amplification of regions characterized by species-specific variation, and thus the most informative for host-plant association within weevils of *R. pilosa* complex. These SPIAs were determined after alignment of *COII* sequences obtained from *de novo* collected specimens of *R. pilosa* species complex associated with *L. vulgaris*, *L. genistifolia* and *L. purpurea*. Primers were tailed with 18 bp long M13 sequences –

302 M13REV (5'-CAGGAAACAGCTATGACC-3') and M13(21) (5'-TGTAACGACGGCCAGT-
303 3') for all forward and reverse primers, respectively. These oligonucleotides were used to improve
304 sequence read length, by sequencing with the adaptors only in both directions (Hernández-Vera *et*
305 *al.*, 2013). PCR conditions for SPIA amplification were the same as described for the full-length
306 mt*COII* amplicon with the exception of extension time and final extension, which were reduced to
307 30 s and 3 min respectively. For phylogenetic analyses we used concatenated SPIAs sequences,
308 which were aligned with homologous regions sampled from *de novo* sequenced specimens from the
309 *R. pilosa* complex. Neighbour-joining trees were generated in MEGA5 software using p-distance
310 method to assess clustering of sequences obtained from dry archival specimens, using *Rhinusa*
311 *antirhini* (Paykull 1800) as an outgroup.

312
313 *Wolbachia* detection. Specimens of distinct mitochondrial lineages of the *R. pilosa* complex
314 associated with different host plants from the genus *Linaria* were tested for *Wolbachia* presence by
315 PCR amplification of the *coxA* housekeeping gene (encodes a catalytic subunit of cytochrome
316 oxidase of the respiratory chain). The *coxA* *Wolbachia* gene was amplified using the primer pair
317 CoxAF1 5' TTGGRGCRATYAACTTTATAG 3' and CoxAR1 5'
318 CTAAAGACTTTKACRCCAGT 3' (Baldo *et al.*, 2006). PCR amplification was performed in a 20-
319 µL reaction volume containing 1 x High Yield Reaction Buffer A with Mg, 1.5 mM MgCl₂, 0.4
320 mM each dNTP, 1 µM each primer, 0.75U of KAPATaq polymerase and 1 µL of DNA template.
321 PCR cycles were carried out using the following thermal profile: 94°C for 2 min, 37 cycles at 94 °C
322 for 30 s, 50°C for 1 min, 72°C for 1 min 30 s, and a final extension at 72°C for 10 min. All PCR
323 products were run on a 1% agarose gel and visualized using ethidium bromide staining. The
324 products of the *coxA* gene were submitted for sequencing in both directions.

325

326

327 Results

328

329 *Gall inducing and host suitability study.* Gall utilization between weevils associated with *L. vulgaris*
330 (= *R. pilosa*) and those associated with *L. genistifolia* was substantially different. In our gall
331 inducing and host suitability tests conducted in no-choice conditions, oviposition of *R. pilosa* on *L.*
332 *vulgaris* resulted in high number of successfully induced galls, while oviposition on *L. genistifolia*
333 resulted in numerous hypersensitive stem reactions which practically blocked gall development and
334 any possibilities of larval development. Only on rare occasions were both gall and larval
335 development recorded on *L. genistifolia* plants after oviposition by *R. pilosa*, and these galls were
336 exclusively induced on plants in a very early phenophase of shoot growth. Thus, mean number of
337 induced galls of *R. pilosa* on *L. genistifolia* was more than ten times lower compared to gall
338 induction on *L. vulgaris*. In a similar manner, weevils sampled from *L. genistifolia* expressed low
339 preference for oviposition and larval development on *L. vulgaris* (Table 3).

340

341 *Mitochondrial COII analyses.* The length of the complete *COII* gene for *R. pilosa* sampled from *L.*
342 *vulgaris* was 690bp, while for *R. brondelii* sampled from *L. purpurea* and *L. genistifolia* yielded
343 693bp and 687bp, respectively. The final 670bp trimmed alignment of the sequences yielded a total
344 of 158 (23.6%) polymorphic nucleotides of which 150 were parsimony informative. The maximum
345 ingroup genetic distance was 16.2% (uncorrected) and all three phylogenetic analyses revealed
346 substantial genetic divergence associated with host plant use (Fig. 3, Table 4). A total of twenty-one
347 different haplotypes were identified across 105 sequenced specimens within the *R. pilosa* complex.
348 The Bayesian information criterion revealed the HKY+G to be the best model to describe the
349 pattern of nucleotide substitution, and this model was employed for the estimation of pairwise
350 genetic distances for a neighbor-joining tree and for Bayesian analysis.

351 *Rhinusa pilosa* populations reared from *L. vulgaris* from Serbia, Hungary and Romania, yielded a
352 total of 14 haplotypes (Fig. 3), and presented an average intraspecific genetic divergence of 4.1%
353 (range 3.7-4.5%). Three haplotypes of *R. brondelii*, with a mean divergence of 0.6% were sampled

354 from *L. purpurea* in Sicily and Calabria (Italy), while divergence among four haplotypes from
355 specimens associated with *L. genistifolia* from Serbia, Hungary and South Russia was 0.3%. The
356 genetic divergence among *R. pilosa* ex *L. vulgaris*, *R. brondelii* haplotypes from *L. purpurea* and
357 samples associated with *L. genistifolia* was 14.2-14.3% and 15.7-16.2% respectively (Table 4). The
358 divergence between *R. brondelii* and samples from *L. genistifolia/dalmatica* was 11%. These results
359 strongly suggest the existence of three taxonomic entities within the *R. pilosa* species complex,
360 which exhibit strict association with the host plants *L. vulgaris* (*R. pilosa* s.s.), *L. purpurea* (= *R.*
361 *brondelii* s.l.) and a third undescribed species associated with *L. genistifolia* and *L. dalmatica*.

362
363 Two mitochondrial lineages associated with *R. pilosa* with genetic divergence of about 4.1% were
364 reared from *L. vulgaris* galls (hereafter referred to haplotype groups Rp-A and Rp-B). Both
365 mitochondrial lineages Rp-A and Rp-B were recorded in nearly all studied populations of *R. pilosa*
366 (Appendix S1). The Rp-A group of haplotypes was dominant (62.8%) in south-eastern Europe
367 (Serbia and Hungary), while Rp-B haplotypes were recorded in 31 (96.9%) out of 32 sequenced
368 specimens originated from Romania. The AT content in both *R. pilosa* haplotype groups ranged
369 from 64-66%, while in haplotypes obtained from *L. purpurea* and *L. genistifolia* AT content was
370 70-71%. Additionally, the Rp-B sequence group from Romania exhibited a higher level of amino
371 acid diversity (up to 4 amino acid substitutions), even though sequenced specimens originated from
372 a single location in Northeast Romania (Popricani, Iași). Moreover, we found very high amino acid
373 diversity between *R. pilosa* haplotype groups Rp-A and Rp-B, yielding from three to six amino-acid
374 differences. The high genetic divergence among haplotypes combined with a high level of amino-
375 acid substitution indicates the possibility that some *COII* gene sequences of *R. pilosa* may represent
376 nuclear mitochondrial pseudogenes (NUMTs) (Song *et al.*, 2008; da Silva *et al.*, 2011). We
377 additionally sequenced the mitochondrial *16S* ribosomal RNA gene (*16S*) for all *R. pilosa COII*
378 haplotypes (Appendix S3; Appendix S4, Table S4-2) in order to compare phylogenetic relatedness
379 using a gene positioned opposite to *COII* gene within the circular structure of mtDNA, and to obtain

380 further support for mitochondrial origin of divergent genotypes within *R. pilosa* populations. To
381 assess this we implemented complementary DNA sequencing protocols to establish the orthology or
382 homology of *COII* gene sequences obtained with TL2-J-3038/TK-N-3782 primers (Appendix S4).
383 Mitochondrial origin of *COII* gene sequences was confirmed for all sequences obtained with TL2-J-
384 3038/TK-N 3782 primers, and these sequences were used for phylogenetic analysis and estimation
385 of divergence time between species from *R. pilosa* complex.

386

387 The median joining network constructed with 21 *COII* haplotypes (Fig. 4), contained only a single
388 ambiguous connection between 4 haplotypes sampled from weevils associated with *L.*
389 *genistifolia/dalmatica*. Within *R. pilosa*, the Rp-A and Rp-B haplotype groups were connected by
390 24 mutational difference, while haplotypes obtained from weevils originated from *L.*
391 *genistifolia/dalmatica* were connected with *R. pilosa* and *R. brondelii* by 92 and 69 mutations,
392 respectively. No reticulations were found in the statistical parsimony network (data not shown), and
393 all haplotype groups corresponding to the Rp-A and Rp-B groups of *R. pilosa*, *R. brondelii* and
394 those associated with *L. genistifolia/dalmatica*, were not connected to the main network at the 95%
395 parsimony connection limit.

396

397 *EF-1 α* analyses. DNA sequencing yielded a total of five nuclear *EF-1 α* haplotypes obtained from
398 specimens associated with all 21 mitochondrial haplotypes from the *R. pilosa* species complex
399 (Appendix S5). From the 634 bp alignment of *EF-1 α* haplotypes (intron was removed from
400 analysis), there were a total of 15 polymorphic nucleotides (2.4%) of which 14 are parsimony
401 informative, with a maximum ingroup genetic distance of 1.6% (uncorrected). A total of three *EF-*
402 *1 α* haplotypes, with a mean divergence of $\approx 0.1\%$ were sampled from the 14 *COII* haplotypes of *R.*
403 *pilosa*. Mean divergence of *EF-1 α* over sequence pairs within Rp-A and Rp-B haplotype groups
404 was 0.2% and 0.1%, respectively (Table 5), while two out of three *R. pilosa* *EF-1 α* haplotypes were
405 common for both Rp-A and Rp-B haplotype groups, revealing a probable absence of genetic sorting

406 between two mitochondrial lineages in regard to *EF-1 α* (Fig. 4). Only one haplotype was recorded
407 for *R. brondelii* and for weevils associated with *L. genistifolia/dalmatica*. The genetic divergence
408 among *R. pilosa* ex *L. vulgaris* (both *COII* genotype groups), *R. brondelii* from *L. purpurea* and
409 samples associated with *L. genistifolia/dalmatica* averaged 1.3 %. The genetic divergences
410 estimated for the *EF-1 α* gene additionally suggest the existence of three well differentiated
411 taxonomic entities within the *R. pilosa* species complex, with strict association regarding their
412 authentic host plants *L. vulgaris* (*R. pilosa* s.s.), *L. purpurea* (= *R. brondelii*) and a third species
413 associated with *L. genistifolia* and *L. dalmatica*. Patterns of relatedness among the nuclear *EF-1 α*
414 sequences were confirmed by median joining (Fig. 4) and statistical parsimony networks analysis
415 (data not shown). Both approaches describe the same relationships among 5 different *EF-1 α*
416 haplotypes within species from *R. pilosa* species complex.

417

418 *Phylogenetic analysis and estimation of divergence time.* All three phylogenetic analyses resulted in
419 near-identical tree topologies that differed in only minor branch arrangements. Three major
420 mitochondrial lineages were clearly distinguished within the *R. pilosa* species complex, each
421 defined host plant association, and each supported by bootstrap values in excess of 90% (Fig. 3).
422 Within *R. pilosa* s.s. two distinct sub-clusters with the high bootstrap support demarcate Rp-A and
423 Rp-B haplotype groups. Two different species, one of which is associated with *L. purpurea* (= *R.*
424 *brondelii* s.l.) and the second associated with *L. genistifolia* and *L. dalmatica* formed sister lineages
425 with an average genetic divergence of about 11%.

426

427 Estimated divergence times for lowest, highest and mean substitution rates are presented in Table 6.
428 According to the mean rate of 0.0152, subs/s/myr, divergence among species within the *R. pilosa*
429 complex (Fig. 3, node II) is estimated to have began approximately 7.2 mya (95%HPD: 4.2-11.1),
430 and the complex diverged from *R. melas* (Boheman, 1838) approximately 10.2 mya (95% HDP:
431 5.4-15.9). The mtDNA lineages associated with *L. purpurea* and *L. genistifolia* (node III) are

432 estimated to have diverged approximately 4.7 mya (95% HPD: 2.5-7.7) while divergence among
433 lineages associated with *L. vulgaris* (node IV) is estimated to have initiated approximately 2.7 mya
434 (95% HPD: 1.1-5.3). Further diversification within *R. pilosa* populations is defined by the existence
435 of two genotype groups, Rp-B (node IV-b) and Rp-A (node IV-a), with estimated ages of
436 diversification of 2.0 Ma (95% HPD: 0.3-3.7) and 0.5 Ma (95% HPD: 0.03-2.2), respectively (Table
437 6).

438

439 *Reassessment of Rhinusa brondelii identity by short mitochondrial fragments.* SPIAs were amplified
440 for 11 of the 15 archival specimens of *R. brondelii* from the MNHN. Ten specimens originated from
441 North Africa, including the neotype of *R. brondelii*. One specimen had location data for south
442 Slovakia. All specimens were considered as *R. brondelii* by Caldara *et al.* (2008). A total of 176 bp
443 were concatenated from the three SPIAs (Appendix S6), and a matrix was constructed with the
444 homologous regions from all haplotypes obtained from *de novo* sequenced specimens within the *R.*
445 *pilosa* complex. A neighbor-joining analysis resulted in a similar tree topology to that derived from
446 full length *COII* sequences (Fig. 5). All North African specimens of *R. brondelii* were clustered
447 with high bootstrap support together with specimens reared from *L. purpurea* galls collected on Mt.
448 Etna, Sicily. Across the three SPIA regions, sequences divergence ranged from 2 to 4 nucleotides,
449 suggesting high relatedness of Sicilian and North African specimens of *R. brondelii* (Table 7).
450 Specimen from South Slovakia clearly clustered with specimens sampled from *L. genistifolia* from
451 southeastern Europe (Fig. 5).

452

453 *Wolbachia detection.* A total of 68 individuals from *R. pilosa* complex (at least 10 from each host
454 associated mitochondrial lineages) were assessed for the presence of *Wolbachia* (Appendix S7).
455 None of the *R. brondelii* specimens (n=11, 6 females, 5 males) from Sicily and Calabria were
456 positive for *Wolbachia*. On the contrary, two different *coxA* *Wolbachia* alleles, here designated as
457 WRp and WRr (Fig. 4), were recorded for *R. pilosa* individuals associated with *L. vulgaris* and

458 weevils associated with *L. genistifolia/dalmatica*, respectively. Out of 47 screened individuals of *R.*
459 *pilosa* from Serbia, Hungary and northeastern Romania, 38 weevils (80.8%, 22 males and 16
460 females) were PCR positive and thus infected uniquely with the *Wolbachia* WRp *coxA* allele. In
461 addition, *Wolbachia* WRp was common within all 8 *R. pilosa* populations and associated with all 14
462 *COII* haplotypes from both lineage Rp-A and Rp-B (Fig. 4) that exhibit deep mitochondrial
463 divergence of 4.1% and 1.6% on *COII* and *16S*, respectively. The *Wolbachia* allele WRr was
464 detected in 6 (3 males, 3 females) out of 10 individuals associated with *L. genistifolia/dalmatica*
465 from Serbia and Hungary. According to the alignment of 402 bp fragment of the *coxA* gene
466 sequences deposited in MLST database (Baldo *et al.*, 2006), WRr is identical to the *coxA*-14 allele,
467 while WRp from *R. pilosa* represents a unique allele showing 99.75% identity to the nearest match
468 (*coxA*-14). Sequences of *Wolbachia* WRp *coxA* and WRr *coxA* alleles are available from GenBank
469 under accession numbers KJ620005 and KJ620006, respectively.

470

471 *Morphological studies.* Body size was a variable character ($p>0.07$) among specimens associated
472 with *L. vulgaris* (= *R. pilosa* s.s.), those associated with *L. purpurea* (= *R. brondelii* s.l.) and
473 specimens reared from *L. genistifolia/dalmatica* plant complex: body length (rostrum excluded) was
474 3.2 ± 0.4 mm (range 2.3-4.1, $n=45$), 3.0 ± 0.4 mm (range 2.3-3.5, $n=30$) and 3.3 ± 0.4 mm (range 2.2-
475 3.8, $n=45$), respectively. Having examined several thousands specimens, particularly *R. pilosa* ex *L.*
476 *vulgaris* and specimens reared from field collected galls on *L. genistifolia/dalmatica*, remarkable
477 variation in body size has been observed, ranging from 1.9-4.2 mm. With regard to colour variation,
478 we observed specimens with pale to dark-brown ground color, including bicolor variation with
479 brown black pronotum and pale brown elytra.

480

481 A morphological character useful for the separation of approximately 80% of the specimens
482 associated either with *L. vulgaris*, *L. purpurea* and *L. genistifolia/dalmatica* is the curvature of the
483 rostrum in the females in lateral view (Fig. 6). The rostrum in *R. pilosa* is abruptly narrowed and

bent along the dorsal margin, then nearly straight to the apex; in *R. brondelii* and specimens reared ex *L. genistifolia/dalmatica* the rostrum is evenly and distinctly curved from base to apex. The width of the rostrum on its conjunction with the head dorsally, is the character that differs most significantly ($p < 0.01$) among *R. pilosa*, *R. brondelii* and specimens associated with the *L. genistifolia/dalmatica* plant complex, measuring 0.23 ± 0.02 ($n=30$), 0.18 ± 0.01 ($n=30$) and 0.20 ± 0.01 ($n=30$), respectively. Another relevant morphological character is the shape of elytra and pronotum in lateral view, which are moderately convex in *R. pilosa* and almost flat in *R. brondelii* and specimens reared from *L. genistifolia/dalmatica*. In addition, the pronotum and elytra in *R. pilosa* s.s. and *R. brondelii* are covered with long hair-like scales (20-40x longer than wide), while these hair-like scales are remarkably recumbent and shorter (10-30x longer than wide) in specimens sampled from with *L. genistifolia/dalmatica* (Fig. 6).

495

496

497 **Taxonomy of the *Rhinusa pilosa* complex**

498

499 According to Caldara *et al.* (2010), *R. pilosa*, together with *R. brondelii* s.l. is a monophyletic
500 group consisting of two closely related species which share two synapomorphic characters: the
501 dorsal vestiture composed of very long seta-like scales and the globose body of the apex of the
502 spermatheca, which are unique within the genus *Rhinusa*. Results obtained from combined
503 morphological, molecular and biological data allow us to recognize the existence of three cryptic
504 species within the *R. pilosa* species complex, strictly associated with their respective host plants
505 from the genus *Linaria*.

506

507 ***Rhinusa pilosa* (Gyllenhal, 1838)**

508 (Figs 1A, 6A-C)

509

510 *Gymnetron pilosum* Gyllenhal, 1838: 763; Brisout de Barneville, 1862: 659; Desbrochers des
511 Loges, 1893: 27. Caldara et al. 2008: 62.

512 *Rhinusa pilosa* (Gyllenhal, 1838). Caldara, 2001: 185; 2013: 142. Caldara et al. 2008: 64. Caldara et
513 al., 2010: 14. Hernández-Vera et al., 2013: 1353.

514

515 *Type series.* This taxon was described from specimens from Podolia (Ukraine) and Magdeburg
516 (Germany), of which a male in the NHRS which fits to original description and redescription by
517 Brisout de Barneville (1862) was designated as the lectotype of *Gymnetron pilosum* Gyllenhal, 1838
518 (for details see Caldara *et al.*, 2008).

519

520 *Diagnosis.* Mean body length (rostrum excluded) 1.9-4.1 mm, for the males 3.0 ± 0.6 (range 1.9-4.1,
521 $n=45$) and for the females 3.3 ± 0.5 (range 2.2-4.1, $n=45$). Integument shining black covered with
522 long hair-like scales (20–40x longer than wide), the longest ones exceeding 0.35 mm. Rostrum in
523 lateral view abruptly narrowed and bent along dorsal margin (especially in females), then straight to
524 apex. Pronotum and elytra in lateral view moderately convex. Legs black, tarsi reddish brown.

525

526 *Distribution.* We can confirm that all the specimens from Sweden, Denmark, Belgium, France,
527 Germany, Hungary, Croatia, Serbia and Romania, discussed by Caldara *et al.* (2008), belong to this
528 species (Fig. 1). In the western Palaearctic, the species is distributed between N43° and N59°
529 latitude. In general, *R. pilosa* is considered as a local and very rare species.

530

531 *Biology.* *Rhinusa pilosa* is a univoltine shoot-galling weevil which overwinters as an adult.
532 Activation of adults occurs in early spring (mid March) coinciding with intensive shoot growth of
533 its host plant *L. vulgaris*. Adults feed intensively for 3-5 days on the tips of young shoots of *L.*
534 *vulgaris* before mating begins, while oviposition starts some 10 days later (beginning of April) and
535 may be prolonged until the end of May, depending upon local environmental conditions. After

536 oviposition, gall development is completed in approximately 8-10 days under laboratory conditions
537 at 23° C, while in field conditions first galls occur during the second week of April. Oviposition
538 occurs on the tips of young growing shoots of *L. vulgaris*. Females typically oviposit 3-6 eggs, but
539 number of ovipositions has been observed to exceed 17 per shoot. Oviposition provokes induction
540 of a globose gall in the apical part of the stem that entirely stops the growth of the attacked shoot
541 (Appendix S8). *Rhinusa pilosa* has three larval instars that feed and complete development within
542 the induced galls. Pupation is also completed within the gall. Eclosed adults feed intensively on
543 remaining gall tissue within the gall before emerging via holes chewed through the gall's outer
544 surface. Newly emerged adults briefly feed externally on the host shoots, then enter into summer
545 aestivation within the soil litter or soil cracks. Summer aestivation is occasionally interrupted by
546 feeding, mainly in the evening and at night. In late autumn, adults are briefly active, feeding on
547 young *L. vulgaris* shoots before entering diapause, sheltering close to host plant. Preferable habitats
548 are sunny exposed slopes and degraded meadows with populations of *L. vulgaris*

549

550 The inquiline weevil species *Rhinusa eversmanni* (Rosenschoeld, 1838) is a gall intruder that
551 utilizes galls induced by *R. pilosa*. This intruder preys upon resident larvae inside galls and is
552 responsible for dramatic reductions in the population density of *R. pilosa*, acquiring proportions of
553 extreme cleptoparasitism. The presence of *R. eversmanni* regularly leads to local extirpation of *R.*
554 *pilosa* populations within 2-3 years (Toševski unpublished data). In field-collected galls induced by
555 *R. pilosa*, a competitive advantage of the former over *R. eversmanni* was rarely observed, i.e. only
556 in galls induced very early during the season. In such circumstances, *R. pilosa* larvae were large
557 enough to kill the larvae of the intruder. However, the majority of successful developments of *R.*
558 *pilosa* occur in galls induced later during the season (second half of May) when oviposition of *R.*
559 *eversmanni* has finished, or is negligible.

560

561 *Remarks.* This species is distinguishable from other species of the *R. pilosa* complex by the rostrum
562 being remarkably bent at the level of the antennal insertion in both sexes. It is the unique species of
563 the group distributed northward in western Palaearctic associated only with *L. vulgaris* as host plant.

564
565
566 ***Rhinusa brondelii* (Brisout de Barneville, 1862)**

567 (Figs 1B, 6D-F)

568
569 *Gymnetron pilosum* Gyllenhal var. *brondelii* Brisout de Barneville, 1862: 659; Caldara et al., 2008:
570 63.

571 *Gymnetron lanuginosum* Wollaston, 1875: 218. Caldara et al., 2008: 63.

572 *Rhinusa brondelii* (Brisout de Barneville, 1862). Caldara et al. 2008: 64 (partim). Caldara et al.,
573 2010: 14. Hernández-Vera et al., 2013: 1353.

574
575
576 *Type series.* Brisout de Barneville described the variety *brondelii* from specimens collected in
577 Algeria (without a more precise locality) differing from typical *G. pilosum* only in its smaller size. It
578 was considered as a subspecies of *G. hispidum* [= *Rhinusa tetra* (Fabricius, 1792)] by Mimeur
579 (1949) and is therefore deemed to carry this rank, according to Art. 45.6.4.1 ICZN, 1999. Because
580 no syntypes of this taxon were found at the MNHN, including in the Brisout de Barneville
581 collection, nor in any other museum, the identity of this taxon was fixed by the designation of a
582 neotype presently deposited in Hoffmann's collection (Algeria, Philipville, currently Skikda) at the
583 MNHN in accordance with the conditions of Art. 75 of the ICZN, 1999 (for details see Caldara *et*
584 *al.*, 2008). We submitted this neotype specimen to DNA extraction, DNA sequencing and
585 phylogenetic analysis by comparing short phylogenetic informative amplicons (SPIAs), which

586 confirmed congruence among all North African, Calabrian and Sicilian species elaborated in present
587 study (see Fig. 5, Table 7, Appendix S2, Appendix S6).

588

589 *Diagnosis.* Mean body length (rostrum excluded) 2.4-3.5 mm, for males 3.0 ± 0.2 (range 2.4-3.4,
590 $n=45$) and for females 3.2 ± 0.2 (range 2.9-3.5, $n=45$). Integument shining black covered with
591 whitish gray, dark brown and black hair-like scales (10-30x longer than wide) the longest ones
592 exceeding 0.35 mm. Rostrum in lateral view evenly curved, in some specimens slightly bent at level
593 of antennal insertion. Pronotum and elytra in lateral view more or less flat. Legs black, tarsi reddish
594 brown.

595

596 *Distribution.* The presence of this species was confirmed from southern Italy including Sicily, and
597 North Africa (Tunisia, Algeria and Morocco) (Fig. 1).

598

599 *Biology.* Univoltine species, the host plant of Italian populations is *Linaria purpurea*. Populations of
600 *R. brondelii* are known from only a few locations in southern Italy and two recently discovered
601 populations on high mountain slopes of Montalto, Aspromonte, Calabria and Mount Etna, Sicily
602 (Appendix S8). Within the locations on Mount Etna, the first galls occurred in early June and,
603 according to the position of the induced galls, oviposition takes place on basal parts of young
604 growing shoots. Mature galls were observed during July and the beginning of August. The majority
605 of adults left galls during August. Adults hibernate in the soil litter or shelters close to a host plant.

606

607 *Remarks.* The molecular study of several museum specimens from North Africa, classified as *R.*
608 *brondelii* from morphology (Caldara *et al.* 2008), confirmed that these specimens were rightly
609 identified. Moreover, the fact that the *COII* sequence of the three specimens from Morocco
610 corresponds with that of the specimens from Tunisia and Algeria suggests that in North Africa there
611 is only one species of this group, and that *R. lanuginosa* is synonymous with *R. brondelii*. The host

612 plant association of the North African populations of *R. brondelii* remains uncertain. The host plant
613 of the specimens from Tunisia could be the widely distributed *Linaria cossoni* Bonn. & G. Barr., a
614 toadflax species which is considered to be conspecific with *L. purpurea* from Italy and Sicily
615 (Viano, 1978; Sutton, 1988). According to Hoffmann (1958) and data obtained from labels attached
616 to archived specimens, *Linaria reflexa* (L.) Chaz. may be a potential host plant for North African
617 populations, but in general further study is needed to determine host range of North African
618 populations of this species.

619

620 ***Rhinusa rara* Toševski & Caldara sp.n.**

621 (Figs 1 C, 6G-I)

622

623 *Holotype*, ♂, SERBIA: Staničenje, ex larva, ex *L. genistifolia*, N43 12.915 E22 30.495, 364 m,
624 7.06.2008, lgt. I.Toševski, DNA-IT350, voucher SRB-35, haplotype rara1, (BMNH). *Paratypes*,
625 SLOVAKIA: ♀, Slovakia mer., Štúrovo, 1.VI.1960, leg. Fremuth, DNA-IT2538 (1♀, MNHN).
626 SERBIA: Aleksinac, ex l., ex *L. genistifolia*, N43 34.495 E21 40.340, 185 m, 2.05.2005, lgt.
627 I.Toševski (20♂, 30♀, RCCM); Sićevo, Niš, ex l., ex *L. genistifolia*, N43 18.698 E22 01.990, 234
628 m, 22.06.2008, lgt. I.Toševski (20♂, 20♀, ITCB; 20♂, 20♀, RCCM; 6♂, 6♀, BMNH; 6♂, 6♀,
629 MNHN; 6♂, 6♀ CABIC; 6♂, 6♀ CBCM); Aleksinac, ex l., ex *L. genistifolia*, N43 34.495 E21
630 40.340, 185 m, 22.06.2008, lgt. I.Toševski (10♂, 10♀, ITCB; 10♂, 10♀, RCCM); Staničenje, Pirot,
631 ex l., ex *L. dalmatica*, N43 13.080 E22 30.218, 379 m, 22.06.2008, (10♂, 10♀, ITC), ex.l., ex *L.*
632 *genistifolia* N43 12.915 E22 30.495, 364 m (10♂, 10♀, ITCB; 10♂, 10♀, RCCM); RUSSIA:
633 Krasnodar, Gulkewitschi, N45 17.882 E40 46.512, 121 m, ex gall, ex *L. genistifolia*, 26.08.2010,
634 lgt. Zoltan Ash (3♂,4♀, ITCB).

635

636 *Etymology*. The Latin adjective *rara*, meaning ‘rare’, emphasizes the very local and scattered
637 distribution of the new species across the eastern part of southern Europe.

638

639 *Description of the holotype.* Male, body length (rostrum excluded) 3.1 mm. Rostrum evenly curved,
640 from base to apex, 0.8 mm long. Antennal segments dark brown. Integument shining black.
641 Pronotum and elytra in lateral view almost flat, densely covered with whitish gray, brown and black
642 hair like scales (10-30x longer than wide), 0.07-0.25 mm long, remarkably recumbent dorsally.
643 Legs black, tarsi reddish-brown.

644

645 *Diagnosis.* Mean body length (rostrum excluded) 2.0-3.7 mm, for males 3.0 ± 0.4 (range 2.0-3.7,
646 $n=45$) and for females 3.2 ± 0.3 (range 2.6-3.8, $n=45$). Rostrum in lateral view evenly curved in both
647 sexes, while rostrum in females remarkably longer than in males. In most specimens integument
648 entirely shining black but in some specimens integument dark-brown to yellowish brown. Pronotum
649 and elytra in lateral view almost flat in both sexes, densely covered with recumbent hair-like scales.

650

651 *Biology.* *Rhinusa rara* is a univoltine shoot galling species overwintering as an adult. The spring
652 activation in the field occurs very early, following activation of its host plant. Just after become
653 active, adults are hidden inside the rosette of the host plant, *L. genistifolia* or *L. dalmatica*, where
654 they feed, copulate and oviposit on new growing shoots. Oviposition occurs at the base of young
655 growing shoots and, because of early oviposition, galls are fully developed by the first week of
656 April (Appendix S8). Usually about 10 eggs are laid per shoot, but some shoots can be induced with
657 20 or more ovipositions. *Rhinusa rara* **sp.n.** has three larval instars and larval development lasts
658 about 45-50 days. The L3 larvae pupate in chambers within the gall tissue. Adults remain inside the
659 gall and feed intensively on gall tissue. After emergence, adults briefly feed externally on host
660 stems. Summer aestivation is interrupted by occasional feeding, mainly in the evening and at night.
661 During the day adults remain hidden in soil litter or soil cracks. In late autumn, adults feed for a
662 brief period before going into hibernation.

663

664 *Rhinusa eversmanni* inhabit galls induced by *R. rara*. The intruder oviposits in fully developed galls
665 of *R. rara* **sp.n.** and its presence may have a strong negative impact on *R. rara* populations. Unlike
666 for galls induced by *R. pilosa* on *L. vulgaris*, where emergence of both weevil species is
667 synchronized and phenology partly overlaps, the attack rate by *R. eversmanni* on *R. rara* galls
668 strongly depends on temperature fluctuation during the early spring. The negative impact of the gall
669 intruder may increase after prolonged period of low temperature during the early spring. The
670 interaction between *R. rara* and its intruder is very important and may perhaps result in local
671 extinction of the gall inducer. To date, all known habitats with significant populations of *R. rara* are
672 calcareous xerothermic slopes of south east exposition. On such habitats, high temperature gradients
673 during early spring favour rapid development of *R. rara* galls and larvae. Thus, because of early
674 oviposition behavior and rapid larval development inside induced galls, most larvae of *R. rara* are
675 already at L3 instars when *R. eversmanni* oviposition begins.

676

677 *Distribution.* Very local and scarce. The species is known only from calcareous regions in Serbia
678 (Sićevo Gorge between town Niš and Pirot), Hungary (Balaton), southern Slovakia (Štúrovo),
679 southern Czechia (Znojmo), Austria (Wien) and southern Russia (Fig. 1).

680

681 *Remarks.* The following examined specimens of *R. rara* **sp.n.**, which were previously identified as
682 *R. brondelii* by Caldara *et al.* (2008), were not included in paratype material: Czech Republic:
683 Moravia, NP. Podyjí, Znojmo 10 km SW Hnanice, 19.VIII.2005, leg. Fremuth (1, JFCH). Slovakia:
684 Štúrovo, 2 km NW Hegyfarok, 17.VI.1958, leg. Fremuth (1, JFCH); Štúrovo 5 km N Kamenica nad
685 Hronom, 15.VI.1958, leg. Fremuth (1, JFCH). Austria: Wien, leg. Reitter (1, DEIM).

686

687 **Key to the species of the *R. pilosa* complex**

- 688 1. Rostrum in lateral view evenly curved in both sexes. Pronotum and elytra almost flat, with
689 integument entirely black, rarely blackish brown or pale-brown, covered with recumbent or sub-
690 erect hair-like scales..... 2
- 691 - Rostrum in lateral view abruptly narrowed and bent along dorsal margin (especially in females),
692 then straight to apex. Pronotum and elytra moderately convex, with integument entirely black,
693 rarely blackish brown or pale-brown, covered bristle and sub-erect hair-like scales.
694 *R. pilosa* (Gyllenhal)
- 695 2. Dorsal integument covered with sub-erect hair-like scales. *R. brondelii* (Brisout de Barneville)
- 696 - Dorsal integument covered with recumbent hair-like scales.
697 *R. rara* Toševski & Caldara **sp.n.**

699 Discussion

700

701 The study of *R. pilosa* was primarily undertaken because of its potential use as a biological control
702 agent for invasive toadflaxes in North America. The taxonomic focus in the early stages of the
703 biological control program was attributed to be *R. hispida* (Brullé, 1832), following literature
704 references describing this taxon as a “well known” gall inducer on *L. vulgaris*. In fact, the identity
705 of this taxon had been uncontroversial for more than one century because of its easy differentiation
706 from all other species of *Rhinusa* by a markedly pilose body (Desbrochers 1893; Reitter, 1907,
707 1916; Hustache, 1931; Hoffmann, 1958; Smreczyński, 1976; Lohse & Tischler, 1983). However,
708 after the examination of a type specimen, Caldara *et al.* (2008) established that *R. hispida sensu*
709 *auctorum* is not the same species as *R. hispida* (Brullé, 1832), which is actually a synonym of
710 *Rhinusa tetra* (Fabricius, 1792). In addition, Caldara *et al.* (2008) stated that under the name *R.*
711 *hispida sensu auctorum* two distinct taxa were confused, *R. pilosa* (Gyllenhal, 1838) and *R.*
712 *brondelii* (Brisout de Barneville, 1862) which are distinguishable from each other by taxonomic,
713 biological and genetic differences. It was concluded that *R. pilosa* is associated with *L. vulgaris* for

larval development, while for *R. brondelii* several species are reported as possible hosts: *L. genistifolia* and *L. dalmatica* for populations in southeastern Europe, and *L. purpurea* and *L. reflexa* for southern Italian and North African populations.

Taxonomical and nomenclatural stabilization of taxa is of great importance in biological control programs and other applied studies. The final decision for the use of an organism as a beneficial agent is related to administrative regulations, which itself implies that taxonomic clarification within a particular group of taxa is of fundamental importance (Goolsby *et al.*, 2006; Gaskin *et al.*, 2011). Thus, species limits among taxa involved in biological control programs is an important issue. In contrast to conclusions based on morphology alone (Hoffmann, 1958; Lohse & Tischler, 1983), studies based on combined morphological, biological and genetic differences have resulted in the assignation of full species status to at least two taxa within the *R. pilosa* group (Caldara *et al.*, 2008). However, further taxonomic differentiation identified within the present study leads to the assignation of full species status of three cryptic taxa, *R. pilosa*, *R. brondelii* and *R. rara* **sp.n.** This conclusion is strongly supported by the biological and ecological properties of these taxa, i.e. strict associations in regard to authentic host species and substantial genetic differences combined with subtle morphological differentiation.

From the point of view of classical biological control, gall-forming organisms are very suitable agents for weed control (Harris & Schorthouse, 1996). The narrow host ranges exhibited within galling taxa is attributed to complex and specific interactions resulting from a close evolutionary association between galling arthropods and their hosts (Hardy & Cook, 2010). In most insect-plant gall systems, the gall growth process is initiated by larval feeding (Dreger-Jauffret & Shorthouse, 1992). In contrast, weevils from the *R. pilosa* group induce fully formed galls prior to larval hatching, which is actually the critical point for further successful larval development. Oviposition plays a key role in stimulating plant tissue responses, leading to gall formation by initiating rapid

740 and specific events followed by the production of new vascular tissue and the occurrence of massive
741 cellular hyperplasy and hypertrophy (Barnewall & De Clerck-Floate, 2012).

742

743 The complexity and specificity among gall inducers with regard to host plant use and specific plant
744 organs galled is substantial and well documented (Manni, 1964; Floate *et al.*, 1996; Abrahamson *et*
745 *al.*, 1998). In our gall inducing and host suitability study tests, oviposition of *R. pilosa* on *L.*
746 *genistifolia* resulted in high number of hypersensitive reactions which practically blocked gall
747 development, and thus any possibility of larval development. On rare occasions we have registered
748 both gall and larval development, but only on *L. genistifolia* plants in very early phenophase of
749 shoot growth.

750

751 The close association between endophagous insects and their host plants may augment the selection
752 pressure imposed by the host (Mopper, 1996), which may lead to diversification, specialization and
753 ecological speciation. The occurrence of distinct host associations in phytophagous insects has been
754 well documented (Feder *et al.*, 1988; Via, 1999; Abrahamson *et al.*, 1998; Via *et al.*, 2000) and
755 hybridization among congeneric plant species, such as within the genus *Linaria*, may create
756 conditions that enhance host shifting of endophagous insects and subsequent ecological divergence
757 (Berlocher & Feder, 2002; Funk *et al.*, 2002). The distribution of the three taxa belonging to the *R.*
758 *pilosa* group follows that of their host–plant distribution. Ecological speciation within the *R. pilosa*
759 group is congruent with speciation events found in other Mecinini associated with *Linaria* spp.,
760 where genetic diversification appears to have been initiated by divergent selection associated with
761 different host plant species (Hernández-Vera *et al.*, 2010; Toševski *et al.*, 2011; Toševski *et al.*,
762 2014).

763

764 On the basis of the genetic data, the *R. pilosa* complex forms two subgroups, as considered by
765 Caldara *et al.* (2010), that is, the *R. pilosa* and *R. brondelii* subgroups. When applying a mean

766 substitution rate of 0.0152, subs/s/myr, the time scale of divergence among the main lineages of the
767 *R. pilosa* species complex seems consistent with geological events in south Europe and North
768 Africa (Table 6). These two subgroups (Fig. 3, node II) are estimated to have diverged from each
769 other approximately 7.2 Ma (95% HPD 4.2–11.1) placing their initial divergence time within a
770 period of dramatic geological events in southern Europe during the late Miocene (11.1–5.3 Ma).
771 The estimated divergence times of 4.7 Ma (95% HPD 2.5–7.7) associated with subsequent
772 speciation within the *R. brondelii* subgroup (node III) coincides with the period after the Messinian
773 salinity crisis, dated between 5.6 and 5.3 Ma (Krijgsman *et al.*, 1999), when a broad range of new
774 land connections existed (De Jong, 1998) and the dispersal of floristic and faunistic elements across
775 the Mediterranean basin (Montreuil, 2008) was possible, prior to rapid inundation during the
776 Zanclean flood (Garcia-Castellanos *et al.*, 2009). Consistent with these events, our results indicate
777 the possibility of biogeographical vicariance for the origin of the two lineages within the *R.*
778 *brondelii* subgroup. However, with no phylogenetic framework for the genus *Linaria*, it is not
779 possible to evaluate if the different host associations of the two lineages were subsequently forced
780 by vicariant host-plant association, or coincident with it, both of which could explain *R. brondelii*
781 associated with *L. purpurea* (South Italy and North Africa) and *R. rara* associated with *L.*
782 *genistifolia/dalmatica* (Southeast Europe). Similar biogeographical scenario of vicariance in the
783 Mediterranean Basin has been documented for Pachydeminae beetles (Sanmartín, 2003), and also in
784 the Mecinini weevils from the *Mecinus heydenii* species complex (Toševski *et al.*, 2013).
785
786 The taxonomic verification of the archival material of *R. brondelii* with SPIAs reveals the unity of
787 Calabrian, Sicilian and North African specimens of this species. If we consider the opinion of Viano
788 (1978) about the conspecific status of the North African *Linaria cossoni* with regard to the Italian *L.*
789 *purpurea*, then the link between Tunisian, Algerian and Sicilian *R. brondelii* becomes more than
790 obvious. There are many documented studies which support intensive transitions of biota during
791 major glaciations events during the Pleistocene and that can explain the origin of vicariant

792 populations distributed in southern Italy and North Africa (Sanmartín, 2003; Maryańska-
793 Nadachowska *et al.*, 2010; Condamine *et al.*, 2013).

794

795 The evolutionary history of *R. pilosa* seems to be less than simple. No differences in morphology
796 were found among studied populations, nor in their biology or phenology. For all studied
797 populations, *L. vulgaris* was found to be the host plant. In contrast, substantial divergence at genetic
798 level was found, with two mtDNA lineages, Rp-A and Rp-B, with estimated divergences of the
799 mitochondrial markers *COII* and *16S*, of 4% and 1.6% respectively. Several authors (e.g. DeSalle *et*
800 *al.*, 1987; Brower, 1994) have proposed that divergence of mitochondrial markers in excess of 2%,
801 or even less (Hebert *et al.*, 2004), may correlate with the existence of cryptic species. In our
802 molecular study we have excluded the presence of nuclear paralogs as a possible explanation of the
803 high mtDNA sequence divergences observed. On the other hand, sequencing of nuclear *EF-1α* gene
804 revealed only three haplotypes within *R. pilosa* populations, two of them (EF-Rp1 and EF-Rp3)
805 common for both mitochondrial lineages Rp-A and Rp-B. Thus, our analyses revealed no
806 association between the distribution of mitochondrial haplotype groups and either geography or
807 nuclear genomic variation, which combined with ecological and morphological factors, argues
808 against the possibility that the different mtDNA haplotype groups may reflect the early stages of
809 speciation, or cryptic speciation. An additional important evidence against cryptic species within *R.*
810 *pilosa* is the frequent appearance of both mtDNA lineages in syntopy, while biological tests
811 revealed no signs of reproductive isolation associated with the two divergent mtDNA lineages
812 (Toševski, unpublished data).

813

814 Deep intraspecific mitochondrial divergence could be correlated with *Wolbachia* infection (Xiao *et*
815 *al.* 2012; Zahiri *et al.*, 2014; Smith *et al.*, 2012), even if the pathway of this mechanism is still not
816 clear (Werren *et al.*, 2008). We detected *Wolbachia* infection within populations of both *R. pilosa*
817 and *R. rara* **sp.n.** However, our data strongly suggest that mitochondrial divergence between Rp-A

818 and Rp-B mitochondrial haplotype groups within *R. pilosa* is not impacted by *Wolbachia* presence,
819 because the common *Wolbachia* allele WRp was recorded in all 14 haplotypes belonging to both
820 mitochondrial groups. This excludes a *Wolbachia* “sweeping effect” on haplotype diversity through
821 cytoplasmatic incompatibility, while it seems more obvious, that abundance of haplotypes is likely
822 driven by a metapopulation effect, or possibly but less probably, the adaptive ability of particular
823 haplotypes *in situ*. Moreover, we have observed equal and balanced sex ratio inside *R. pilosa*
824 populations during mass rearing experiments with field collected *R. pilosa* on behalf of biological
825 control programme between 2004-2012 (Toševski, unpublished data). This excludes the three other
826 distinct reproductive phenotypes caused by *Wolbachia*, i.e. male killing, feminization and
827 parthenogenesis. Nevertheless, it is obvious that populations of *R. pilosa* consist of deeply divergent
828 mitochondrial haplotype groups, with unusually high divergences between haplotypes within groups
829 (e.g. Rp-B). Based on the evidence, the most plausible explanation is that the divergence *R. pilosa*
830 mitochondrial lineages evolved in allopatry, with subsequent admixture proceeding from secondary
831 contact between previously separated populations (Futuyma, 2005).

832

833 According to our phylogenetic dating analysis, the two mtDNA lineages of *R. pilosa* (Fig. 3, node
834 IV) diverged from each other approximately 2.7 Ma (95% HPD 1.1-5.3) placing their initial
835 divergence time at the beginning of Pleistocene (≈ 2.6 Ma). Further sequence divergence within Rp-
836 B (node IV-b) was estimated to have initiated 2.0 Ma ((95% HPD 0.3–3.7) which overlaps with the
837 first major glacial event during Galesian age (2.6-1.8 Ma), while for genotype group Rp-A (node
838 IV-a) subsequent genetic differentiation occurs much later at approximately 0.5 Ma (95% HPD
839 0.03–2.2) during Ionian age (0.780-0.126 Ma).

840

841 Our molecular data suggest that within glacial and inter-glacial cycles during the Pleistocene,
842 populations of *R. pilosa* probably experienced successive periods of isolation that lead to
843 accumulation of genetic differences among isolated populations. It is difficult to suggest probable

ice-age refugia for populations of *R. pilosa*, but according to the present distribution, it seems that refugia for post glacial expansion of Rp-B genotypes could be associated with more easterly located populations. Recent studies also suggest the possible existence of extra-Mediterranean ice-age refugia, distributed across central Europe (Stewart & Lister, 2001; Schmitt, 2007; Provan & Bennett, 2008). Estimated intra-lineage divergence time and the distribution of lineage Rp-A suggest that this group may have originated from Balkan refugia, where haplotypes from lineage Rp-A are predominant (>62%). The genetic heterogeneity of *R. pilosa* populations may be explained by dispersal and range expansion during interglacial or stadial periods, resulting in the missing of divergent genetic variation resulting from previously isolated allopatric populations. The notion of repeated glacial contraction and range fragmentation, followed by expansion and coalescence of eastern and southern populations of *R. pilosa* during subsequent interglacial periods is in accord with Coope (1994) and Abellán *et al.* (2011) who argues for intensive range changes of insects during the Quaternary by comparing fossil records and contemporary distributions.

857

The validation of taxon boundaries, especially inside groups burdened with taxonomic uncertainty, is one of the more important and fundamental issues within biological control programs. Our study of the *R. pilosa* species complex revealed the existence of three cryptic taxa, where two out of the three, *R. pilosa* and *R. rara* **sp.n.**, are proposed as candidate agents for the biological control of invasive toadflaxes in North America, *L. vulgaris* and *L. dalmatica*, respectively. Deep genetic divergence between *R. pilosa* genotypes Rp-A and RR-B may be of particular interest for further evolutionary studies related with this species complex, however increased taxon and molecular sampling across the western Palearctic is required for a proper evaluation.

866

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Table 1. Primer pairs used for the amplification of mitochondrial and nuclear genes in the *Rhinusa pilosa* complex.

primer pairs	primer position	primer sequence (5'... 3')	Amplicon length ^a (bp)	sequencing primer
TL2-J-3038 TK-N 3782	tRNA-Leu tRNA-Lys	TAATATGGCAGATTAGTGCATTGGA GAGACCATTACTTGCTTTTCAGTCATCT	788	TL2-J-3038
16Scf1* 16Sbr1*	16S rRNA 16S rRNA	TTGTACCTTGTGTATCAGGGTT AGATAGAAACCAACCTGGCTTAC	≈1000	16br1
EF1-Bf EF-Br	<i>EF-1α</i>	AGAACGTGAACGTGGTATCA CTTGGAGTCACCAGCTACATAACC	≈1070	EF1-Bf, EF-Br

* primers designed for this study

^a product length without primers

Table 2. Primers used for the amplification of short mitochondrial cytochrome oxidase subunit II (mtCOII) fragments.

Primer ^a	Primer sequence (without tails)	Amplicon length (without tails) (bp)	Sequencing primer
248Ft	5' TACTATACATTTTAGATGA 3'	88	M13REV
248Rt	5' TCGGAGTATTCATAGGATCA 3'		M13(-21)
450Ft	5' TCGAGTGATTGTTACATC 3'	85	M13REV
450Rt	5' TCGTCCTGGTGTTCATCA 3'		M13(-21)
515Ft	5' TTGATGCAACACCAGGACGATTAAA 3'	131	M13REV
515Rt	5' AGAAACTGCCTCAATAACAATAGGTA 3'		M13(-21)

^a Primers named according to the position of the forward primer accounted from the beginning of the mtCOII gene in *Rhinusa pilosa* species group.

Table 3. No-choice oviposition, gall inducing and larval development tests with *Rhinusa pilosa* originating from *Linaria vulgaris* and *L. genistifolia*.

host plant	No. of female	No. of plants	total no. of oviposition	No. of galls induced	No. of adults emerged	Mean no. of galls $\pm$ SD/plants (range)	Mean no. of adults $\pm$ SD/plants (range)	% of oviposition resulting development
<i>Rhinusa pilosa</i> ex <i>Linaria vulgaris</i>								
<i>Linaria vulgaris</i> (control)	30	172	1363	356	1037	2.1 $\pm$ 1.6 (0-11)	6.0 $\pm$ 5.0 (0-34)	76.1
<i>L. genistifolia</i> (test)	18	93	594 (476 HSR*)	20	37	0.2 $\pm$ 0.7 (0-5)	0.4 $\pm$ 1.4 (0-7)	6.2
<i>populations</i> ex <i>Linaria genistifolia/dalmatica</i>								
<i>L. genistifolia</i> (control)	28	149	1505	269	1192	1.8 $\pm$ 1.5 (0-6)	8.0 $\pm$ 6.7 (0-34)	79.2
<i>Linaria vulgaris</i> (test)	13	63	160	34	73	0.5 $\pm$ 0.8 (0-4)	1.2 $\pm$ 1.9(0-7)	45.6

*Hypersensitive stem reaction

Table 4. Average mitochondrial DNA cytochrome oxidase subunit II (*COII*) divergence based on pairwise analysis (p-distance method) among haplotypes of the *Rhinusa pilosa* complex, grouped according to their host plant affiliation.

Sequences group of mtCOII gene	d ₁ (S.E.)	d ₂ (S.E.)	P (S.E.)			
			1	2	3	4
1. ex <i>L. vulgaris</i> genotype Rp-A		0.002 (0.001)	-	(0.007)	(0.013)	(0.013)
2. ex <i>L. vulgaris</i> genotype Rp-B	0.084 (0.006)	0.007 (0.002)	0.041	-	(0.013)	(0.013)
3. ex <i>L. purpurea</i>		0.006 (0.002)	0.143	0.142	-	(0.012)
4. ex <i>L. genistifolia/dalmatica</i>		0.003 (0.001)	0.157	0.162	0.110	-

(d₁) divergence over all sequence pairs; (d₂) divergence over sequence pairs within groups; (P) p-distance over sequence pairs between groups; (S.E.) standard error.

Table 5. Average nuclear DNA elongation factor-1 α gene divergence based on the pairwise analysis (p-distance method) among haplotypes of the *Rhinusa pilosa* complex, grouped according to their host plants. Affiliation of *Rhinusa pilosa* COII genotype groups Rp-A and Rp-B are indicated in parentheses.

Sequences group of <i>EF-1α</i> gene	d ₁ (S.E.)	d ₂ (S.E.)	P (S.E.)			
			1	2	3	4
1. ex <i>L. vulgaris</i> (Rp-A)		0.002 (0.001)	-	(0.001)	(0.004)	(0.004)
2. ex <i>L. vulgaris</i> (Rp-B)	0.008 (0.002)	0.001 (0.001)	0.002	-	(0.004)	(0.004)
3. ex <i>L. purpurea</i>		0	0.014	0.013	-	(0.004)
4. ex <i>L. genistifolia/dalmatica</i>		0	0.014	0.013	0.012	-

(d₁) divergence over all sequence pairs; (d₂) divergence over sequence pairs within groups; (P) p-distance over sequence pairs between groups; (S.E.) standard error.

Table 6. Estimated time to the most recent common ancestor (mrca) for host associated mitochondrial lineages of the *Rhinusa pilosa* complex expressed as mean value according to: (A) lowest (0.006 subs/site/myr), (B) highest (0.0245 subs/site/myr) and (C) mean substitution rate (0.01525 subs/site/myr) with 95% highest posterior density interval (in brackets).

Node	description of node	mean value in Ma		
		A	B	C
I	root of tree	26.0 (16.9-35.8)	6.4 (3.9-9.2)	10.2 (5.4-15.9)
II	mrca of <i>R. pilosa</i> complex (ingroup)	18.4 (13.1-24.3)	4.5 (2.9-6.3)	7.2 (4.2-11.1)
III	mrca of lineage <i>L. purpurea</i> / <i>L. genistifolia</i>	12.1 (7.3-17.4)	3.0 (1.7-4.4)	4.7 (2.5-7.7)
IV	mrca of lineage <i>L. vulgaris</i>	6.7 (3.2-12.0)	1.6 (0.7-2.8)	2.7 (1.1-5.3)
IV-b	mrca of lineage Rp-B genotype	4.9 (0.7-8.4)	1.2 (0.2-2.0)	2.0 (0.3-3.7)
IV-a	mrca of lineage Rp-A genotype	1.2 (0.09-5.3)	0.3 (0.02-1.3)	0.5 (0.03-2.2)

Table 7. Interspecific informative sites between *Rhinusa brondelii* specimens from Sicily associated with *Linaria pupurea* and specimens from North Africa (MNHN). The position of short phylogenetically informative amplicons (SPIAs) in regard to the complete length of the *COII* gene is shown in brackets.

origin of specimens	SPIA 1 (248-335)		SPIA 2 (450-534)			SPIA 3 (515-645)	
	276*	306*	495*	504*	582*	600*	603*
Mt Etna, Sicily, (haplotypes bron1, bron2, bron3)	G	C	C	A	G	T	A
Museum specimens							
2532 Alger, NEOTYPE	A	T	.	.	.	.	G
2533 Alger	A	T	.	.	.	.	G
2534 Alger	A	T	.	.	.	.	.
2535 Alger	A	T	.	.	A	C	.
2539 Tunisie, Souk-el-Arba	A	T	.	.	-	.	.
2541 Morocco, Rabat	A	T	.	G	A	.	.
2542 Morocco, Safi	A	T	.	G	A	.	.
2543 Morocco, Safi	A	T	.	G	A	.	.
2544 Tunisia, Souk-el-Arba	A	T	T	.	.	.	.
2545 Tunisia, Souk-el-Arba	A	T	.	.	.	.	.

* Informative sites within the *COII* gene

Fig. 1. Confirmed localities for *Rhinusa pilosa* (Gyllenhal, 1838), *R. brondelii* (Brisout de Barneville, 1862) and populations of *R. rara* sp.n. associated with *Linaria genistifolia* according to archived material (dots) and newly collected specimens (stars) in the western Palaearctic.

Fig. 2. Schematic representation of primer locations for the amplification of mt*COII* region of the *R. pilosa* species complex. Primers marked with an asterisk (*) were used for the amplification of short phylogenetically informative amplicons of mt*COII* gene from archival specimens. Dashed arrows indicate position of primers used for amplification of the pseudogene (explained in Appendix S4). Not drawn to scale.

Fig. 3. Bayesian phylogenetic tree inferred from 670 bp of mtDNA *COII* gene sampled from the *Rhinusa pilosa* species complex from different toadflax taxa. Bayesian posterior probabilities are shown above branches, maximum parsimony and neighbour-joining bootstrap support values are indicated below branches in that order (only values for key nodes are shown). Roman numerals and lower case letters refer to estimated divergence times given in Table 6. Arabic numerals refer to host plant-associated lineages: (1) *Linaria vulgaris*; (2) *L. genistifolia* and *L. dalmatica*; (3) *L. purpurea*.

Fig. 4. Median joining network obtained from *COII* and *EF-1 α* sequences of the *Rhinusa pilosa* species complex. Circle sizes are proportional to haplotype frequency. Colours correspond to the mitochondrial lineages associated with different host plants. Red dots are median vectors and black dots represent missing haplotypes. Numbers in brackets above/beside solid broken line represents number of mutations connecting mitochondrial lineages. *EF-1 α* haplotypes EF-Rp1 and EF-Rp3 are associated with more than one

mitochondrial lineage. Mitochondrial haplotypes associated with *Wolbachia* strains WRp and WRr are circled with a dashed line.

Fig. 5. Neighbour-joining tree (p-distance) constructed in MEGA5 using concatenated short phylogenetically informative amplicons (SPIAs) of the mtDNA *COII* gene from 11 archival specimens deposited at MNHN and corresponding sequences from all 21 *COII* haplotypes identified within *Rhinusa pilosa* species complex using *de novo* collected material. Specimens from Sicily reared ex *L. purpurea* and the neotype of *R. brondelii* are marked in bold. Bootstrap support values for main nodes are shown.

Fig. 6. Lateral view of the species of the *Rhinusa pilosa* species complex: (A-C) *R. pilosa* (A) male, (B) female, (C) detail of female rostrum; (D-F) *R. brondelii* (D) male, (E) female, (F) detail of female rostrum; (G-I) *R. rara* **sp.n.**, (G) male, (H) female, (I) detail of female rostrum.