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

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

This published work has been registered in ZooBank, http://zoobank.org/urn:lsid:zoobank.org:pub:EEDD6248-01DB-4B4A-B79D-C5606393E3AA.

Introduction

The weevil genus *Rhinusa* Stephens (Curculionidae, Mecinini) is composed of approximately 40 species, all with a Palearctic distribution (Reitter, 1907; Caldara, 2001, 2013). Based on morphological characters, all species from the genus *Rhinusa* have been divided into 10 species groups (Caldara *et al.*, 2010). A phylogenetic analysis based on morphology suggests that the genus *Rhinusa* is monophyletic and sister to the Palaearctic species of the genus *Gymnetron* Schoenherr, 1825 (Caldara, 2001). However, a recent comprehensive molecular phylogenetic analysis does not support monophyly for either genus, but does suggest an origin for all taxa from an ancestral *Gymnetron* species from southern Africa. It was concluded that subsequent range expansion from southern Africa was followed by extensive species radiation in the western Palaearctic, with the earliest divergence estimated to date back to circa 27 million years ago (mya) (Hernández-Vera *et al.*, 2013).

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

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

The recent wide use of molecular techniques substantially improves possibilities for reliable taxonomic clarification in problematic taxa, delineation of cryptic species, estimation of population structure and genetic variability (Tay *et al.*, 2012; Krosch *et al.*, 2013), and fast species identification, particularly in applied studies (Gaskin *et al.*, 2011). Most frequently, the target genetic marker of choice in such studies is mitochondrial. Although broadly exploited, particularly after the barcode initiative (Hebert *et al.*, 2003), analyses based only on mitochondrial markers may lead to erroneous results because of relatively frequent unintended co-amplification of nuclear pseudogenes (numts). This is usually of concern when deep mitochondrial divergence is observed within populations that are considered as a unique taxon (Bensasson *et al.*, 2001; Song *et al.*, 2008; Kindler *et al.*, 2012). Recently, more comprehensive phylogenetic studies have shown that deeper mitochondrial divergence within populations of particular arthropod species can be driven by the presence of parasitic *Wolbachia* and its ability to manipulate with mitochondrial DNA (mtDNA) of the host (Werren *et al.*, 2008; Smith *et al.*, 2012). Due to these potential complications, precautionary measures and a holistic approach should be undertaken when choosing appropriate markers and methods of phylogenetic analyses for cryptic species determination and evaluation.

Over the last 10 years, the potential of stem gall-forming weevils as potential biological agents for the control of invasive toadflaxes has been the subject of several studies (e.g. Toševski *et al.*, 2005). Host suitability studies of the stem-galling weevils from the *R. pilosa* species group (Toševski *et al.*, 2012) have suggested highly host-specific interactions between weevil populations and their respective toadflax host, with elements of cryptic speciation, previously suggested by Caldara *et al.* (2008). These findings reinforce the need to evaluate the taxonomic placement of populations of *Rhinusa* associated with stem gall induction in other toadflax species in the western Palaearctic. This is of particular importance, as the consequences will underpin decisions regarding these taxa as potential biocontrol agents. Here we present the results of morphological, biological and genetic studies on stem gall-inducing weevils within the *R. pilosa* species group sampled from toadflaxes in Europe to better understand their taxonomic status, nomenclatural positions and the approximate temporal framework for their evolution and historical biogeography.

Materials and methods

Acronyms

The collections housing the material studied in this revision are abbreviated as follows (with their curators in parentheses):

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

Specimens examined

This study is based on a previous examination of the type specimen series and archived specimens published by Caldara

et al. (2008), and newly collected material for morphological and genetic analysis (Appendix S1). Several hundred specimens were examined in order to determine the geographical distribution of the studied taxa. Specimens for molecular analysis sampled from *L. vulgaris*, *L. genistifolia* and *L. purpurea* were reared from galled plants collected in Hungary (*L. vulgaris*, *L. genistifolia*), Romania (*L. vulgaris*), Serbia (*L. vulgaris*, *L. genistifolia*, *L. dalmatica*), southern Russia (*L. genistifolia*), and southern Italy (*L. purpurea*) (Fig. 1; Appendix S1). In addition, the neotype of *Gymnetron pilosum* var. *brondelii* Brisout de Barneville, 1862 (des. Caldara) and 14 archival voucher specimens deposited in the MNHN and previously ascribed to *R. brondelii* by Caldara *et al.* (2008) were submitted for DNA extraction and sequencing to reassess their taxonomic status (Appendix S2).

Morphological study

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

Gall-inducing and host suitability study

Gall induction studies were carried out with weevil populations associated with *L. vulgaris* (= *R. pilosa*) and populations of weevils associated with *L. genistifolia* and *L. dalmatica* as part of host specificity tests performed with both species within the framework of their assessment within biological control studies (Toševski *et al.*, 2012). In two trials, post-hibernated females sampled from *L. vulgaris* and *L. genistifolia* were set up for oviposition on their respective field host plants (control series) and the field host plants of the other population (test series). In accordance with their biology and phenology, the no-choice gall induction test was set up at the beginning of April when oviposition begins on both host species (Toševski *et al.*, 2005). During testing, females were transferred to fresh potted plants every 3–4 days for oviposition. Each plant was checked for oviposition and gall induction, while the number of oviposition marks per induced gall was also recorded. Galls were dissected for adult development during the first week of July. The number of induced galls, total number of ovipositions, and successful development to adult were recorded from each plant. The significance of the results from the larval development experiment was assessed by total and mean development rate on control and test plants and the percentage of recorded ovipositions that resulted in development to adult.

Material for DNA study

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

DNA extraction

Individual weevils were punctured at the second thoracic sternite and total DNA was extracted using the Qiagen Dneasy® Blood & Tissue Kit (Hilden, Germany), according to the manufacturer's instructions. The same procedure was used to extract DNA from the dry neotype specimen of *R. brondelii* and 14 other specimens of *R. brondelii* originating from North Africa, all deposed in the MNHN collection in Paris.

PCR amplification and sequencing

The mitochondrial *COII* was amplified using the primers TL2-J-3038 (Emerson *et al.*, 2000) and TK-N 3782 (Harrison Laboratory, Cornell University, Ithaca, NY, USA) (Table 1). Amplification reactions were performed in a 20 µL final reaction volume containing Kapabiosystems High Yield Reaction Buffer A with 1.5 mM $MgCl_2$ (1×), an additional 3.5 mM $MgCl_2$, 0.8 mM of each dNTP, 0.75 µM of each primer, 0.75 U of KAPA*Taq* DNA polymerase (Kapa Biosystems, Inc., Woburn, MA, USA) and 1 µL of DNA extract. Polymerase chain reactions (PCRs) were carried out in a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany) applying the following thermal steps: 95°C for 5 min (initial denaturation), and 40 cycles at 95°C for 1 min, 1 min at 45°C (annealing), 72°C for

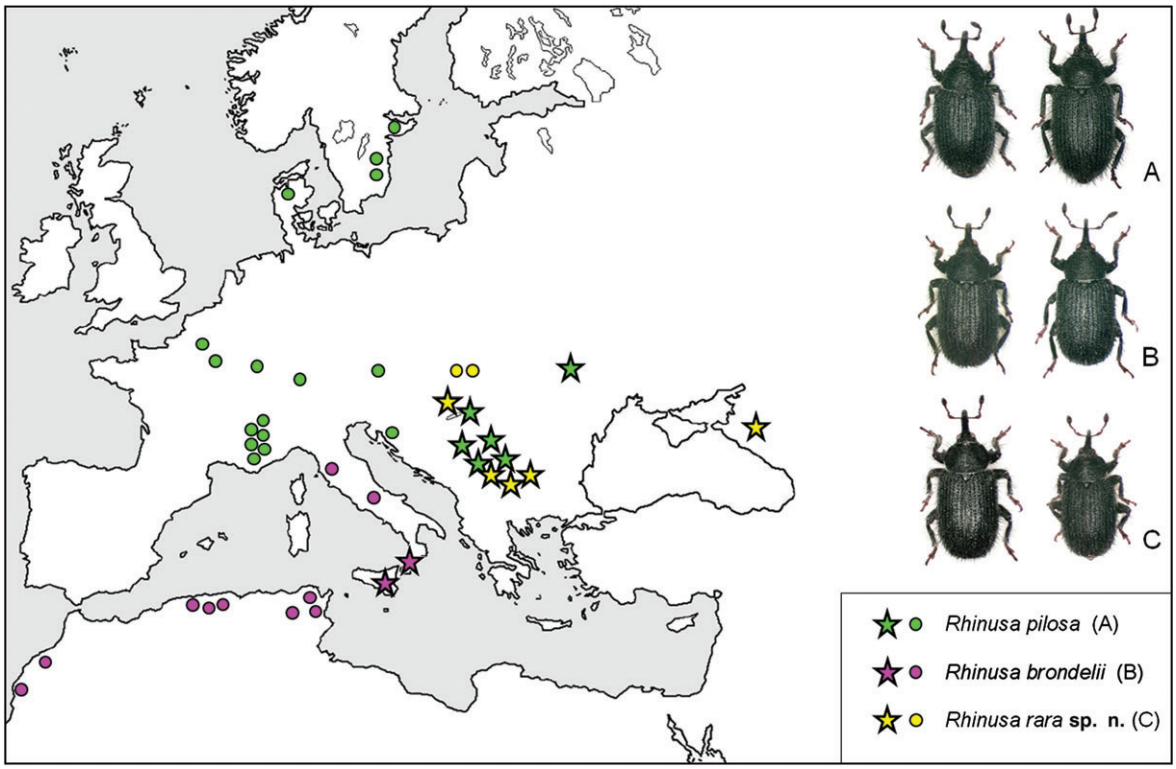

Fig. 1. Confirmed localities for *Rhinusa pilosa* (Gyllenhal, 1838), *Rhinusa brondelii* (Brisout de Barneville, 1862) and populations of *Rhinusa rara* **sp.n.** associated with *Linaria genistifolia/dalmatica* according to archived material (dots) and newly collected specimens (stars) in the western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

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	tRNA-Leu	TAATATGGCAGATTAGTGCATTGGA	788	TL2-J-3038
TK-N 3782	tRNA-Lys	GAGACCATTACTTGCTTTCAGTCATCT		
16Scf1[b]	*16S* rRNA	TTGTACCTTGTGTATCAGGGTT	≈1000	16Sbr1
16Sbr1[b]		AGATAGAAACCAACCTGGCTTAC		
EF1-Bf	*EF-1α*	AGAACGTGAACGTGGTATCA	≈1070	EF1-Bf, EF-Br
EF-Br		CTTGGAGTCACCAGCTACATAACC		

[a]Product length without primers.
[b]Primers designed for this study.

2 min, and a final extension at 72°C for 10 min. The mitochondrial *16S* was amplified using the primers 16Scf1 and 16Sbr1 (Table 1). Reactions were performed with 1× High Yield Reaction Buffer A with Mg, 2.25 mM $MgCl_2$, 0.6 mM of each dNTP, 0.5 μM of each primer, 1U of KAPATaq polymerase and 1 μL of DNA template in a 20 μL final volume. PCRs were carried out using the following thermal profile: 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 extension at 72°C for 7 min. Amplifications of the nuclear gene elongation factor-1α (*EF-1α*), were performed with the primers EF1-Bf and EF-Br (Hernández-Vera *et al.*, 2013; Table 1). Reactions were performed in a 20 μL final reaction volume under the same reaction and thermal conditions as for *16S* 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 *16S* were obtained with the reverse primer only. All specimens were sequenced in both directions for the *EF-1α* amplicon. Sequences are available from GenBank under accession numbers KJ420489–KJ420509 for the *COII* gene (Appendix S1), KJ420513–KJ420523 for the *16S* ribosomal RNA gene (Appendix S3) and KJ620000–KJ620004 for the *EF-1α* gene (Appendix S5).

Evolutionary tree construction and estimation of divergence times

Sequences were edited with FINCHTV v.1.4.0 (http://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 neighbour-joining trees were generated in PAUP* version 4.0b10 (Swofford, 2002) using the evolutionary model of nucleotide substitution that best fits the data, determined with JMODELTEST version 2.1.1 (Darriba *et al.*, 2012). Five hundred bootstrap replicates were performed to assess branch support of the resulting tree topologies. Bayesian analyses were carried out with 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).

In the absence of geological and/or fossil calibration points related to weevils from the genus *Rhinusa*, we adopted a Bayesian approach to estimate the age of the most recent common ancestor (mrca) of the whole group and the inferred lineages. Mitochondrial rates have been proposed for arthropods in the range 1.2–4.9% pairwise divergence per million years (myr) (DeSalle *et al.*, 1987; Brower, 1994; Caccone & Sbordoni, 2001). To take this reported variance into account, we applied per lineage rates of 0.006 and 0.0245 substitutions per site per myr (subs/s/myr) which correspond to 1.2 and 4.9% pairwise divergence per myr, respectively. Furthermore, we applied a mean lineage rate of 0.0152 subs/s/myr, which is equivalent to 3.05% pairwise divergence per myr, following Cicconardi *et al.* (2010), who demonstrated the mean substitution rate for the *COII* gene within Coleoptera to approximate the rate across the Pancrustacea. The mean lineage rate of 0.0152 subs/s/myr applied in our analysis closely matches the rate of 0.0134 subs/site/myr proposed by Pons *et al.* (2010), and 0.0177 subs/site/myr proposed by Papadopoulou *et al.* (2010). In order to estimate divergence times among genetic lineages within the *R. pilosa* species group, we used BEAST version 1.7.4 (Drummond *et al.*, 2012). For our analyses we applied a random local clock model (Drummond & Suchard, 2010) with the average number of substitutions per site across the tree averaged to be 1.525%/myr. The initial substitution model parameter values were the Hasegawa–Kishino–Yano model with gamma distribution (HKY + G), selected according to the results of JMODELTEST, with unconstrained prior distributions. A separate demographic model of constant population size was applied, in the form of a coalescent prior, to clades conforming to genetic variation within host-associated lineages, and a Yule tree prior was used for the basal branches connecting these. Input files were generated with BEAUTI version 1.7.4 (Drummond *et al.*, 2012). Two runs were conducted for the lowest, highest and mean substitution rates, each consisting of 30 000 000 generations, and sampling every 1000 generations. For each of the three rate values, the two runs were combined after checking sampling, mixing and convergence of Markov chains to a stationary distribution. Estimated lineage divergence times were summarized and expressed as mean values in myr 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-1α* 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 (http://www.fluxus-engineering.com) keeping the parameter $\varepsilon = 0$, 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 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, and these were 85, 88 and 131 bp in length, respectively (Table 2, Fig. 2). Primers were positioned within the *COII* gene sequence for the amplification of regions characterized by species-specific variation, and which were thus the most informative for host–plant association within weevils of the *R. pilosa* complex. These SPIAs were determined after alignment of *COII* sequences obtained from de novo collected specimens of the *R. pilosa* species complex associated with *L. vulgaris*, *L. genistifolia* and *L. purpurea*. Primers were tailed with 18-bp-long M13 sequences – M13REV (5′-CAGGAAACAGCTATGACC-3′) and M13(21) (5′-TGTAAAACGACGGCCAGT-3′) – for all forward and reverse primers, respectively. These oligonucleotides were used to improve sequence read length, by sequencing with the adaptors only in both directions (Hernández-Vera *et al.*, 2013). PCR conditions for SPIA amplification were the same

Table 2. Primers used for the amplification of short mitochondrial cytochrome oxidase subunit II (mt*COII*) 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′ TCGTCCTGGTGTTGCATCA 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 mt*COII* gene in *Rhinusa pilosa* species group.

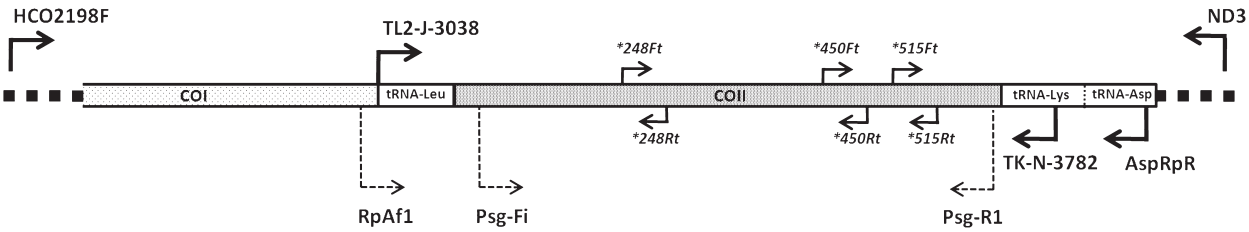

Fig. 2. Schematic representation of primer locations for the amplification of the mitochondrial cytochrome oxidase subunit II gene (mt*COII*) region of the *Rhinusa pilosa* species complex. Primers marked with an asterisk (*) were used for the amplification of short phylogenetically informative amplicons of the 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.

as described for the full-length mt*COII* amplicon, with the exception of extension time and final extension, which were reduced to 30 s and 3 min respectively. For phylogenetic analyses, we used concatenated SPIA sequences, which were aligned with homologous regions sampled from de novo sequenced specimens from the *R. pilosa* complex. Neighbour-joining trees were generated in MEGA5 software using the p-distance method to assess clustering of sequences obtained from dry archival specimens, using *Rhinusa antirhini* (Paykull 1800) as an outgroup.

Wolbachia detection

Specimens of distinct mitochondrial lineages of the *R. pilosa* complex associated with different host plants from the genus *Linaria* were tested for *Wolbachia* presence by PCR amplification of the *coxA* housekeeping gene (encodes a catalytic subunit of cytochrome oxidase of the respiratory chain). The *coxA Wolbachia* gene was amplified using the primer pair CoxAF1 5′ TTGGRGCRATYAACTTTATAG 3′ and CoxAR1 5′ CTAAAGACTTTKACRCCAGT 3′ (Baldo *et al.*, 2006). PCR amplification was performed in a 20 μL reaction volume containing 1× High Yield Reaction Buffer A with Mg, 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 1 μM of each primer, 0.75U of KAPA*Taq* polymerase and 1 μL of DNA template. PCR cycles were carried out using the following thermal profile: 94°C for 2 min, 37 cycles at 94°C 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 products were run on a 1% agarose gel and visualized using ethidium bromide staining. The products of the *coxA* gene were submitted for sequencing in both directions.

Results

Gall-inducing and host suitability study

Gall utilization between weevils associated with *L. vulgaris* (=*R. pilosa*) and those associated with *L. genistifolia* was substantially different. In our gall-inducing and host suitability tests conducted in no-choice conditions, oviposition of *R. pilosa* on *L. vulgaris* resulted in a high number of successfully induced galls, while oviposition on *L. genistifolia* resulted in numerous hypersensitive stem reactions that practically blocked gall development and any possibilities of larval development. Only on rare occasions were both gall and larval development recorded on *L. genistifolia* plants after oviposition by *R. pilosa*, and these galls were exclusively induced on plants in a very early phenophase of shoot growth. Thus, the mean number of induced galls of *R. pilosa* on *L. genistifolia* was more than 10 times lower than with gall induction on *L. vulgaris*. In a similar manner, weevils sampled from *L. genistifolia* expressed low preference for oviposition and larval development on *L. vulgaris* (Table 3).

Mitochondrial COII analyses

The length of the complete *COII* gene for *R. pilosa* sampled from *L. vulgaris* was 690 bp, while those for *R. brondelii* sampled from *L. purpurea* and *L. genistifolia* yielded 693 and 687 bp, respectively. The final 670 bp trimmed alignment of the sequences yielded a total of 158 (23.6%) polymorphic nucleotides of which 150 were parsimony-informative. The maximum ingroup genetic distance was 16.2% (uncorrected) and all three phylogenetic analyses revealed substantial genetic

Table 3. No-choice oviposition, gall-inducing and larval development tests with *Rhinusa pilosa* originating from *Linaria vulgaris* and *Linaria 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 ± SD/plants (range)	Mean no. of adults ± SD/plants (range)	% of oviposition resulting development
Rhinusa pilosa* ex *Linaria vulgaris								
L. vulgaris (control)	30	172	1363	356	1037	2.1 ± 1.6 (0–11)	6.0 ± 5.0 (0–34)	76.1
L. genistifolia (test)	18	93	594 (476 HSR[a])	20	37	0.2 ± 0.7 (0–5)	0.4 ± 1.4 (0–7)	6.2
Populations* ex *Linaria genistifolia/dalmatica								
L. genistifolia (control)	28	149	1505	269	1192	1.8 ± 1.5 (0–6)	8.0 ± 6.7 (0–34)	79.2
L. vulgaris (test)	13	63	160	34	73	0.5 ± 0.8 (0–4)	1.2 ± 1.9 (0–7)	45.6

[a]Hypersensitive stem reaction.

divergence associated with host plant use (Fig. 3, Table 4). A total of 21 different haplotypes were identified across 105 sequenced specimens within the *R. pilosa* complex. The Bayesian information criterion revealed the HKY+G to be the best model to describe the pattern of nucleotide substitution, and this model was employed for the estimation of pairwise genetic distances for a neighbour-joining tree and for Bayesian analysis.

Rhinusa pilosa populations reared from *L. vulgaris* from Serbia, Hungary and Romania yielded a total of 14 haplotypes (Fig. 3), and presented an average intraspecific genetic divergence of 4.1% (range 3.7–4.5%). Three haplotypes of *R. brondelii*, with a mean divergence of 0.6%, were sampled from *L. purpurea* in Sicily and Calabria (Italy), while divergence among four haplotypes from specimens associated with *L. genistifolia* from Serbia, Hungary and southern Russia was 0.3%. The genetic divergences among *R. pilosa* ex *L. vulgaris*, *R. brondelii* haplotypes from *L. purpurea* and samples associated with *L. genistifolia* were 14.2–14.3 and 15.7–16.2% respectively (Table 4). The divergence between *R. brondelii* and samples from *L. genistifolia/dalmatica* was 11%. These results strongly suggest the existence of three taxonomic entities within the *R. pilosa* species complex, which exhibit strict association with the host plants *L. vulgaris* (*R. pilosa* s.s.), *L. purpurea* (= *R. brondelii* s.l.) and a third undescribed species associated with *L. genistifolia* and *L. dalmatica.*

Two mitochondrial lineages associated with *R. pilosa* with genetic divergence of about 4.1% were reared from *L. vulgaris* galls (hereafter referred to haplotype groups Rp-A and Rp-B). Both mitochondrial lineages Rp-A and Rp-B were recorded in nearly all studied populations of *R. pilosa* (Appendix S1). The Rp-A group of haplotypes was dominant (62.8%) in south-eastern Europe (Serbia and Hungary), while Rp-B haplotypes were recorded in 31 (96.9%) out of 32 sequenced specimens originated from Romania. The AT content in both *R. pilosa* haplotype groups ranged from 64% to 66%, while in haplotypes obtained from *L. purpurea* and *L. genistifolia*, the AT content was 70–71%. Additionally, the Rp-B sequence group from Romania exhibited a higher level of amino acid diversity (up to four amino acid substitutions), even though sequenced specimens originated from a single location in north-east Romania (Popricani, Iaşi). Moreover, we found very high amino acid diversity between *R. pilosa* haplotype groups Rp-A and Rp-B, yielding from three to six amino-acid differences. The high genetic divergence among haplotypes combined with a high level of amino-acid substitution indicates the possibility that some *COII* gene sequences of *R. pilosa* may represent nuclear mitochondrial pseudogenes (NUMTs) (Song *et al.*, 2008; da Silva *et al.*, 2011). We additionally sequenced the mitochondrial *16S* ribosomal RNA gene (*16S*) for all *R. pilosa COII* haplotypes [Appendices S3 and S4 (Table S4-2)] in order to compare phylogenetic relatedness using a gene positioned opposite to the *COII* gene within the circular structure of mtDNA, and to obtain further support for the mitochondrial origin of divergent genotypes within *R. pilosa* populations. To assess this, we implemented complementary DNA sequencing protocols to establish the orthology or homology of *COII* gene sequences obtained with TL2-J-3038/TK-N-3782 primers (Appendix S4). The mitochondrial origin of *COII* gene sequences was confirmed for all sequences obtained with TL2-J-3038/TK-N 3782 primers, and these sequences were used for phylogenetic analysis and estimation of divergence time between species from the *R. pilosa* complex.

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

EF-1α analyses

DNA sequencing yielded a total of five nuclear *EF-1α* haplotypes obtained from specimens associated with all 21 mitochondrial haplotypes from the *R. pilosa* species complex (Appendix S5). From the 634 bp alignment of *EF-1α* haplotypes (intron was

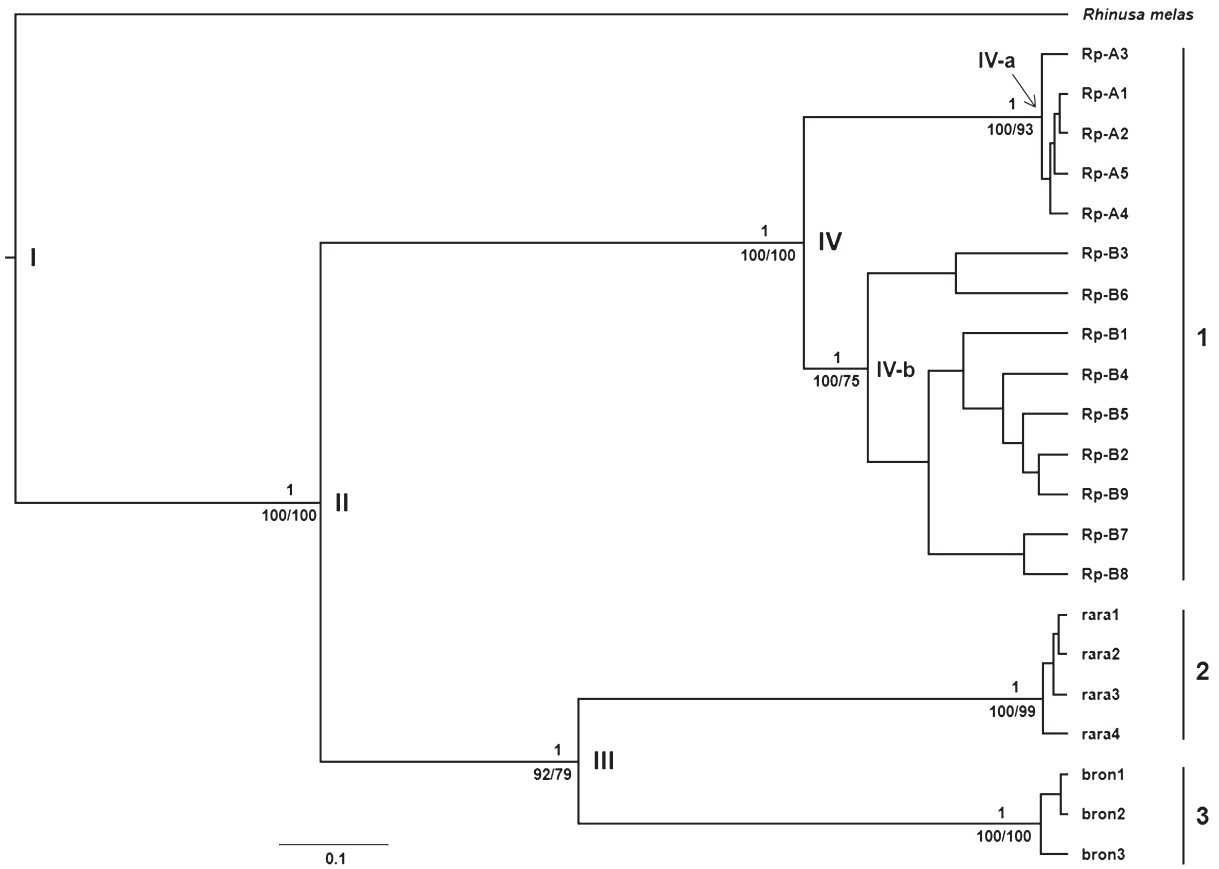

Fig. 3. Bayesian phylogenetic tree inferred from 670 bp of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit II (*COII*) gene sampled from the *Rhinusa pilosa* species complex from different toadflax taxa. Bayesian posterior probabilities are shown above branches, and maximum parsimony and neighbour-joining 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, *Linaria genistifolia* and *Linaria dalmatica*; 3, *Linaria purpurea*.

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.

			P (SE)			
Sequences group of mtCOII gene	d_1 (SE)	d_2 (SE)	1	2	3	4
1. ex *Linaria vulgaris* genotype Rp-A	0.084 (0.006)	0.002 (0.001)	–	(0.007)	(0.013)	(0.013)
2. ex *L. vulgaris* genotype Rp-B		0.007 (0.002)	0.041	–	(0.013)	(0.013)
3. ex *Linaria purpurea*		0.006 (0.002)	0.143	0.142	–	(0.012)
4. ex *L. genistifolia/dalmatica*		0.003 (0.001)	0.157	0.162	0.110	–

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

removed from analysis), there were a total of 15 polymorphic nucleotides (2.4%), of which 14 are parsimony-informative, with a maximum ingroup genetic distance of 1.6% (uncorrected). A total of three *EF-1α* haplotypes, with a mean divergence of ≈0.1% were sampled from the 14 *COII* haplotypes of *R. pilosa.* The mean divergences of *EF-1α* over sequence pairs within Rp-A and Rp-B haplotype groups were 0.2 and 0.1%, respectively (Table 5), while two out of three *R. pilosa EF-1α* haplotypes were common for both Rp-A and Rp-B haplotype groups, revealing a probable absence of genetic sorting between two mitochondrial lineages with regard to *EF-1α* (Fig. 4). Only one haplotype was recorded for *R. brondelii* and for weevils associated with *L. genistifolia/dalmatica*. The genetic divergence among *R. pilosa* ex *L. vulgaris* (both *COII* genotype groups), *R. brondelii* from *L. purpurea* and samples associated with *L. genistifolia/dalmatica* averaged 1.3%. The genetic divergences estimated for the *EF-1α* gene additionally suggest the existence of three well-differentiated taxonomic entities within

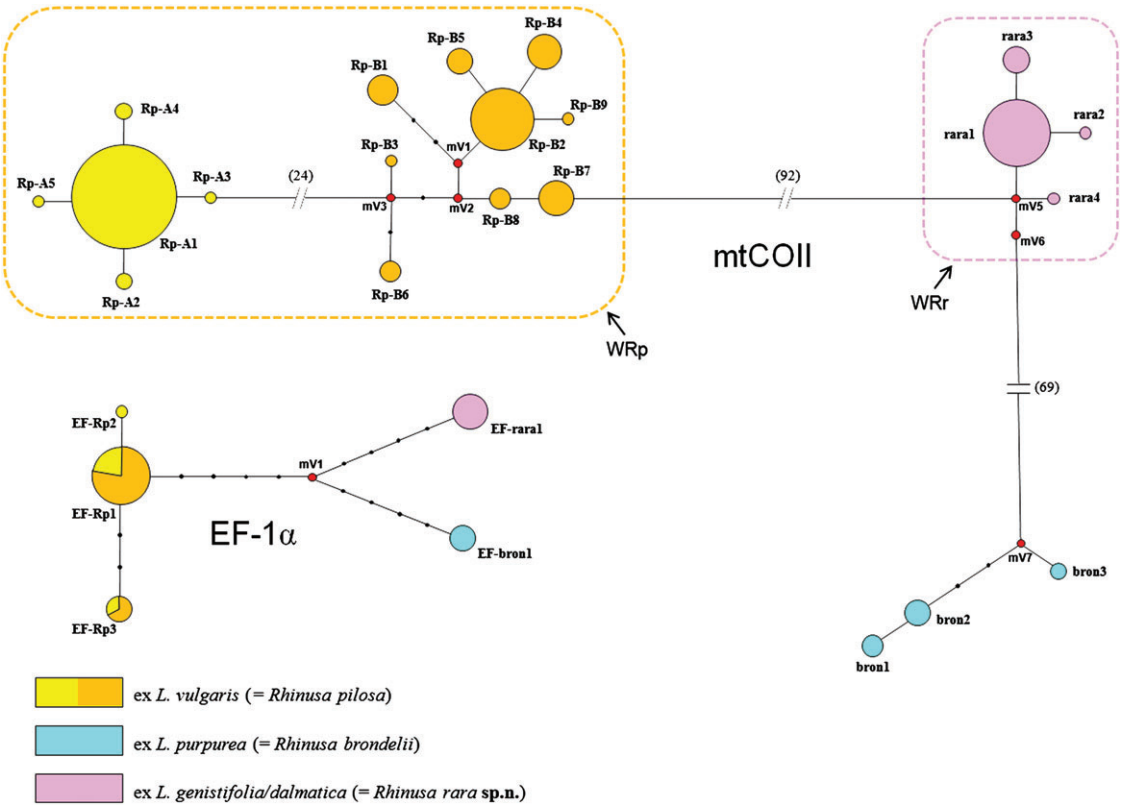

Fig. 4. Median joining network obtained from cytochrome oxidase subunit II (*COII*) and elongation factor-1α (*EF-1α*) sequences of the *Rhinusa pilosa* species complex. Circle sizes are proportional to haplotype frequency. Colours correspond to the mitochondrial lineages associated with different host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

the *R. pilosa* species complex, with strict association regarding their authentic host plants *L. vulgaris* (*R. pilosa* s.s.), *L. purpurea* (= *R. brondelii*) and a third species associated with *L. genistifolia* and *L. dalmatica*. Patterns of relatedness among the nuclear *EF-1α* sequences were confirmed by median joining (Fig. 4) and statistical parsimony network analysis (data not shown). Both approaches describe the same relationships among five different *EF-1α* haplotypes within species from the *R. pilosa* species complex.

Phylogenetic analysis and estimation of divergence time

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

Estimated divergence times for lowest, highest and mean substitution rates are presented in Table 6.

According to the mean rate of 0.0152 subs/s/myr, divergence among species within the *R. pilosa* complex (Fig. 3, node II) is estimated to have began approximately 7.2 mya (95% HPD, 4.2–11.1), and the complex diverged from *R. melas* (Boheman, 1838) approximately 10.2 mya (95% HPD, 5.4–15.9). The mtDNA lineages associated with *L. purpurea* and *L. genistifolia* (node III) are estimated to have diverged approximately 4.7 mya (95% HPD, 2.5–7.7), while divergence among lineages associated with *L. vulgaris* (node IV) is estimated to have initiated approximately 2.7 mya (95% HPD, 1.1–5.3). Further diversification within *R. pilosa* populations is defined by the existence of two genotype groups, Rp-B (node IV-b) and Rp-A (node IV-a), with estimated ages of diversification of 2.0 mya (95% HPD, 0.3–3.7) and 0.5 mya (95% HPD, 0.03–2.2), respectively (Table 6).

Reassessment of Rhinusa brondelii identity by short mitochondrial fragments

Short phylogenetically informative amplicons were amplified for 11 of the 15 archival specimens of *R. brondelii* from

Table 5. Average nuclear DNA elongation factor-1α 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 *R. pilosa* cytochrome oxidase subunit II (*COII*) genotype groups Rp-A and Rp-B are indicated in parentheses.

			P (SE)			
Sequences group of *EF-1α* gene	d_1 (SE)	d_2 (SE)	1	2	3	4
1. ex *Linaria vulgaris* (Rp-A)	0.008 (0.002)	0.002 (0.001)	–	(0.001)	(0.004)	(0.004)
2. ex *L. vulgaris* (Rp-B)		0.001 (0.001)	0.002	–	(0.004)	(0.004)
3. ex *Linaria purpurea*		0	0.014	0.013	–	(0.004)
4. ex *Linaria genistifolia/dalmatica*		0	0.014	0.013	0.012	–

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

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 the lowest [0.006 substitutions per site per million years (subs/site/myr)] (A), highest (0.0245 subs/site/myr) (B) and mean substitution rates (0.01525 subs/site/myr) (C) with 95% highest posterior density interval (in brackets).

		Mean value (myr)		
Node	Description of node	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 *R. pilosa* complex (ingroup)	18.4 (13.1–24.3)	4.5 (2.9–6.3)	7.2 (4.2–11.1)
III	mrca of lineage *Linaria purpurea/ Linaria genistifolia*	12.1 (7.3–17.4)	3.0 (1.7–4.4)	4.7 (2.5–7.7)
IV	mrca of lineage *Linaria vulgaris*	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)

the MNHN. Ten specimens originated from North Africa, including the neotype of *R. brondelii*. One specimen had location data for south Slovakia. All specimens were considered as *R. brondelii* by Caldara *et al.* (2008). A total of 176 bp were concatenated from the three SPIAs (Appendix S6), and a matrix was constructed with the homologous regions from all haplotypes obtained from de novo sequenced specimens within the *R. pilosa* complex. A neighbour-joining analysis resulted in a similar tree topology to that derived from full-length *COII* sequences (Fig. 5). All North African specimens of *R. brondelii* were clustered with high bootstrap support together with specimens reared from *L. purpurea* galls collected on Mount Etna, Sicily. Across the three SPIA regions, sequences divergence ranged from two to four nucleotides, suggesting high relatedness of Sicilian and North African specimens of *R. brondelii* (Table 7). Specimen from southern Slovakia clearly clustered with specimens sampled from *L. genistifolia* from south-eastern Europe (Fig. 5).

Wolbachia detection

A total of 68 individuals from the *R. pilosa* complex (at least 10 from each host-associated mitochondrial lineage) were assessed for the presence of *Wolbachia* (Appendix S7). None of the *R. brondelii* specimens ($n = 11$, six females, five males) from Sicily and Calabria were positive for *Wolbachia*. On the contrary, two different *coxA Wolbachia* alleles, here designated as WRp and WRr (Fig. 4), were recorded for *R. pilosa* individuals associated with *L. vulgaris* and weevils associated with *L. genistifolia/dalmatica,* respectively. Out of 47 screened individuals of *R. pilosa* from Serbia, Hungary and north-eastern Romania, 38 weevils (80.8%, 22 males and 16 females) were PCR-positive and thus infected uniquely with the *Wolbachia* WRp *coxA* allele. In addition, *Wolbachia* WRp was common within all eight *R. pilosa* populations and associated with all 14 *COII* haplotypes from both lineages Rp-A and Rp-B (Fig. 4), which exhibit deep mitochondrial divergence of 4.1% and 1.6% on *COII* and *16S*, respectively. The *Wolbachia* allele WRr was detected in six (three males, three females) out of 10 individuals associated with *L. genistifolia/dalmatica* from Serbia and Hungary. According to the alignment of the 402-bp fragment of the *coxA* gene sequences deposited in the MLST database (Baldo *et al.*, 2006), WRr is identical to the *coxA*-14 allele, while WRp from *R. pilosa* represents a unique allele showing 99.75% identity to the nearest match (*coxA*-14). Sequences of *Wolbachia* WRp *coxA* and WRr *coxA* alleles are available from GenBank under accession numbers KJ620005 and KJ620006, respectively.

Morphological studies

Body size was a variable character ($P > 0.07$) among specimens associated with *L. vulgaris* (= *R. pilosa* s.s.), those associated with *L. purpurea* (= *R. brondelii* s.l.) and specimens reared from the *L. genistifolia/dalmatica* plant complex: body lengths (rostrum excluded) were 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 2.2–3.8, $n = 45$), respectively. Having examined several

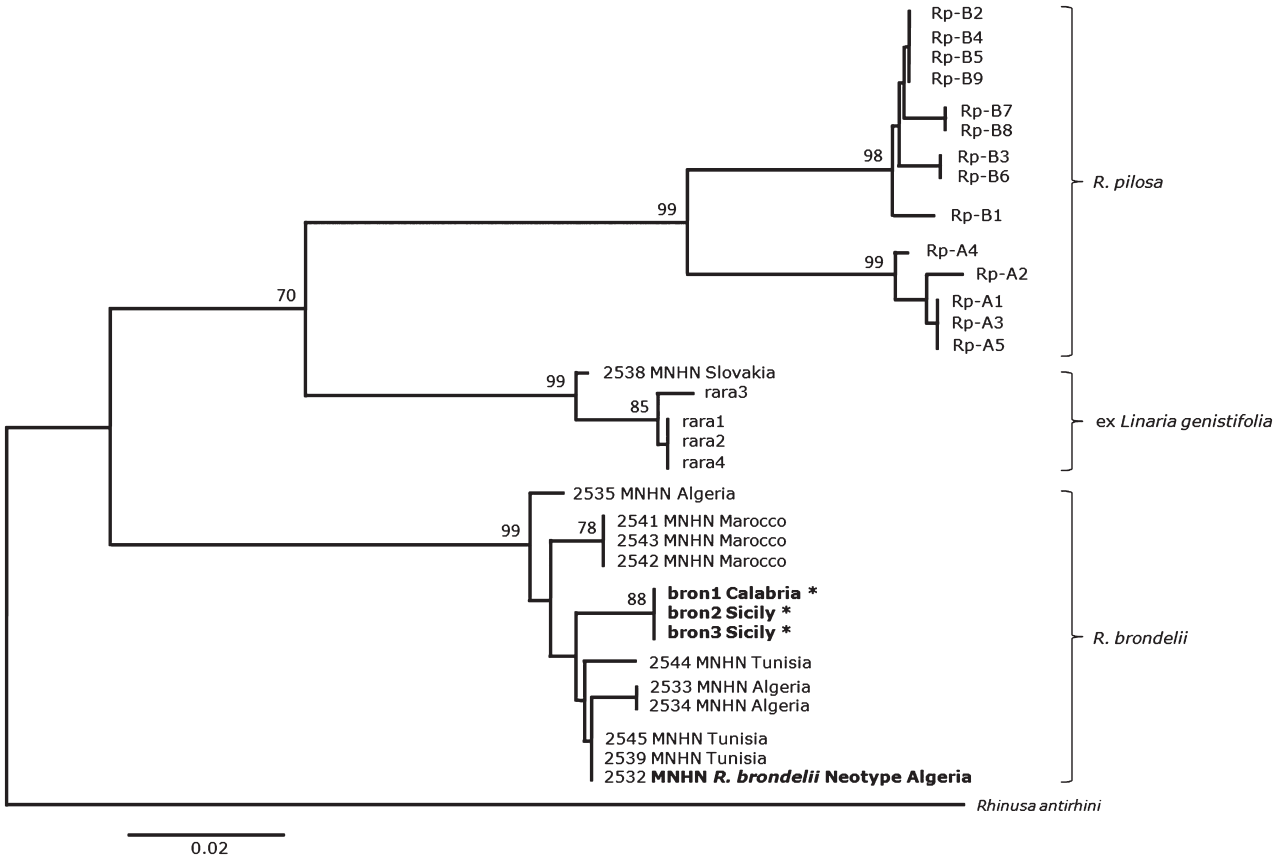

Fig. 5. Neighbour-joining tree (p-distance) constructed in MEGA5 using concatenated short phylogenetically informative amplicons (SPIAs) of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit II (*COII*) gene from 11 archival specimens deposited at Muséum National d'Histoire Naturelle, Paris, France, and corresponding sequences from all 21 *COII* haplotypes identified within *Rhinusa pilosa* species complex using *de novo* collected material. Specimens from Sicily reared ex *Linaria purpurea* and the neotype of *Rhinusa brondelii* are marked in bold. Bootstrap support values for main nodes are shown.

thousand specimens, particularly *R. pilosa* ex *L. vulgaris* and specimens reared from field-collected galls on *L. genistifolia/dalmatica*, a remarkable variation in body size was observed, ranging from 1.9 to 4.2 mm. With regard to colour variation, we observed specimens with pale to dark brown ground colour, including bicolour variation with brown black pronotum and pale brown elytra.

A morphological character useful for the separation of approximately 80% of the specimens associated with *L. vulgaris*, *L. purpurea* or *L. genistifolia/dalmatica* is the curvature of the rostrum in females in the lateral view (Fig. 6). The rostrum in *R. pilosa* is abruptly narrowed and bent along the dorsal margin, and 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 (20–40× longer than wide), while these hair-like scales are remarkably recumbent and shorter (10–30× longer than wide) in specimens sampled from with *L. genistifolia/dalmatica* (Fig. 6).

Taxonomy of the *R. pilosa* complex

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

Table 7. Interspecific informative sites between *Rhinusa brondelii* specimens from Sicily associated with *Linaria pupurea* and specimens from North Africa (Muséum National d'Histoire Naturelle, Paris, France). The position of short phylogenetically informative amplicons (SPIAs) in relation to the complete length of the cytochrome oxidase subunit II (*COII*) gene is shown in brackets.

Origin of specimens	SPIA 1 (248–335) 276[a]	306[a]	SPIA 2 (450–534) 495[a]	504[a]	582[a]	SPIA 3 (515–645) 600[a]	603[a]
Mount 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	–	–	–	–	–

[a]Informative sites within the *COII* gene.

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

(Figs 1A, 6A–C).

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

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

Type series. This taxon was described from specimens from Podolia (Ukraine) and Magdeburg (Germany), of which a male in the NHRS that fits the original description and redescription by Brisout de Barneville (1862) was designated as the lectotype of *Gymnetron pilosum* Gyllenhal, 1838 (for details see Caldara *et al.*, 2008). For non-type material examined see Appendix S1.

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

Distribution. We can confirm that all the specimens from Sweden, Denmark, Belgium, France, Germany, Hungary, Croatia, Serbia and Romania, discussed by Caldara *et al.* (2008), belong to this species (Fig. 1). In the western Palaearctic, the species is distributed between 43°N and 59°N latitude. In general, *R. pilosa* is considered as a local and very rare species.

Biology. Rhinusa pilosa is a univoltine shoot-galling weevil that overwinters as an adult. Activation of adults occurs in early spring (mid-March), coinciding with intensive shoot growth of its host plant *L. vulgaris*. Adults feed intensively for 3–5 days on the tips of young shoots of *L. vulgaris* before mating begins, while oviposition starts some 10 days later (beginning of April) and may be prolonged until the end of May, depending upon local environmental conditions. After oviposition, gall development is completed in approximately 8–10 days under laboratory conditions at 23°C, while in field conditions first galls occur during the second week of April. Oviposition occurs on the tips of young growing shoots of *L. vulgaris*. Females typically oviposit three to six eggs, but the number of ovipositions has been observed to exceed 17 per shoot. Oviposition provokes induction of a globose gall in the apical part of the stem that entirely stops the growth of the attacked shoot (Appendix S8). *Rhinusa pilosa* has three larval instars that feed and complete development within the induced galls. Pupation is also completed within the gall. Eclosed adults feed intensively on remaining gall tissue within the gall before emerging via holes chewed through the gall's outer surface. Newly emerged adults briefly feed externally on the host shoots, and then enter into summer aestivation within the soil litter or soil cracks. Summer aestivation is occasionally interrupted by feeding, mainly in the evening and at night. In late autumn, adults are briefly active, feeding on young *L. vulgaris* shoots before entering diapause, sheltering close to the host plant. Preferable habitats are sunny exposed slopes and degraded meadows with populations of *L. vulgaris.*

The inquiline weevil species *Rhinusa eversmanni* (Rosenschoeld, 1838) is a gall intruder that utilizes galls induced by *R. pilosa*. This intruder preys upon resident larvae inside galls and is responsible for dramatic reductions in the population density of *R. pilosa*, acquiring proportions of extreme cleptoparasitism. The presence of *R. eversmanni* regularly leads to local extirpation of *R. pilosa* populations within 2–3 years (I. Toševski, unpublished data). In field-collected galls induced by *R. pilosa*, a competitive advantage of the former over *R. eversmanni* was rarely observed, i.e. only in galls induced very

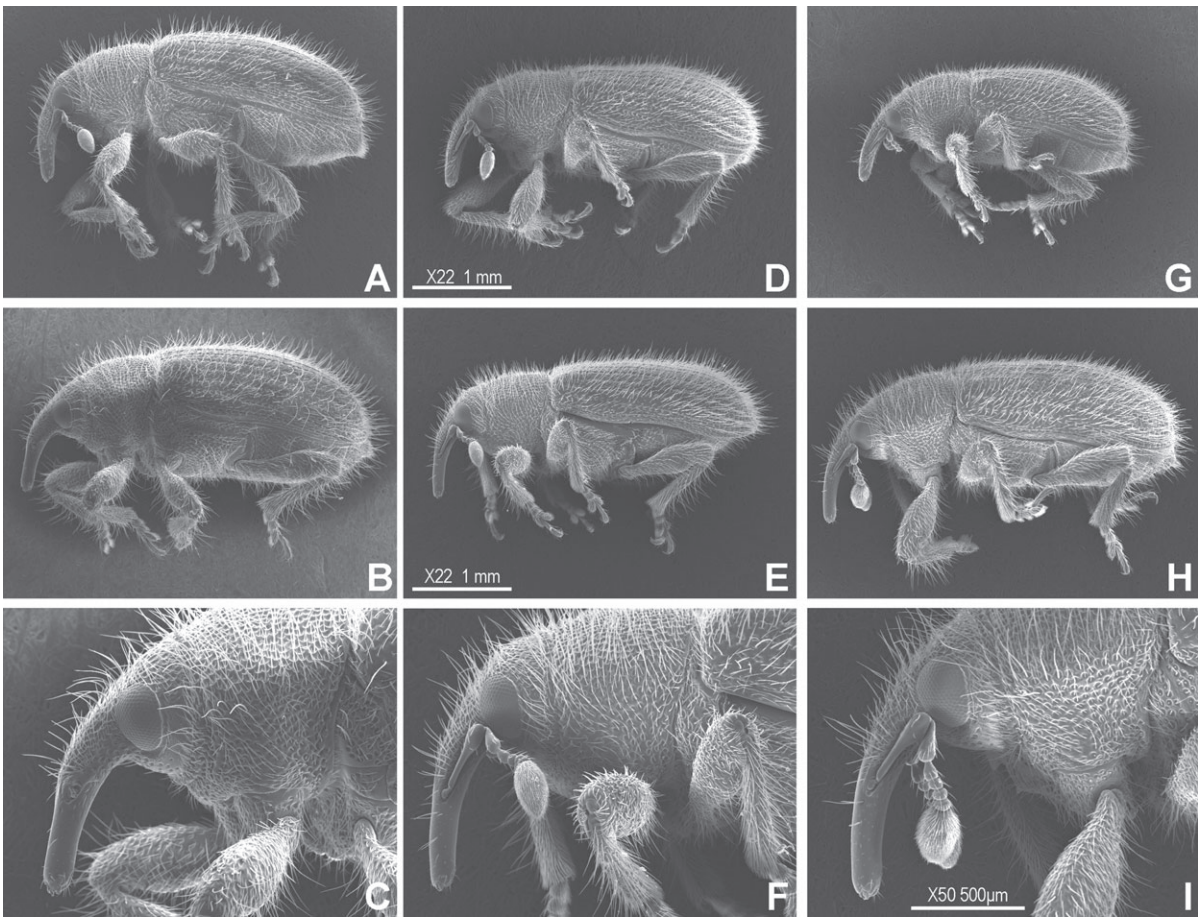

Fig. 6. Lateral view of the species of the *Rhinusa pilosa* species complex. (A–C) *R. pilosa* male (A), female (B), detail of female rostrum (C). (D–F) *Rhinusa brondelii* male (D), female (E), detail of female rostrum (F). (G–I) *Rhinusa rara* **sp.n.** male (G), female (H), detail of female rostrum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

early during the season. In such circumstances, *R. pilosa* larvae were large enough to kill the larvae of the intruder. However, the majority of successful developments of *R. pilosa* occur in galls induced later during the season (second half of May) when oviposition of *R. eversmanni* has finished, or is negligible.

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

***Rhinusa brondelii* (Brisout de Barneville, 1862)**
(Figs 1B, 6D–F).

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

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

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

Type series. Brisout de Barneville described the variety *brondelii* from specimens collected in Algeria (without a more precise locality) differing from typical *G. pilosum* only in its smaller size. It was considered as a subspecies of *G. hispidum* [=*Rhinusa tetra* (Fabricius, 1792)] by Mimeur (1949) and is therefore deemed to carry this rank, according to Art. 45.6.4.1 ICZN, 1999. Because no syntypes of this taxon were found at the MNHN, including in the Brisout de Barneville collection, or in any other museum, the identity of this taxon was fixed by the designation of a neotype presently deposited in Hoffmann's collection (Algeria, Philipville, currently Skikda) at the MNHN in accordance with the conditions of Art. 75 of the ICZN, 1999 (for details, see Caldara *et al.*, 2008). We submitted this neotype specimen to DNA extraction, DNA sequencing and phylogenetic analysis by comparing short phylogenetic informative amplicons (SPIAs), which confirmed congruence among all North African, Calabrian and Sicilian species elaborated in present study (see Fig. 5, Table 7, Appendices S2 and S6). For non-type material examined, see Appendix S1.

Diagnosis. Mean body length (rostrum excluded) 2.4–3.5 mm, for males 3.0 ± 0.2 mm (range 2.4–3.4, $n = 45$)

and for females 3.2 ± 0.2 mm (range 2.9–3.5, $n = 45$). Integument shining black covered with whitish gray, dark brown and black hair-like scales (10–30× longer than wide) the longest ones exceeding 0.35 mm. Rostrum in lateral view evenly curved, in some specimens slightly bent at level of antennal insertion. Pronotum and elytra in lateral view more or less flat. Legs black, tarsi reddish brown.

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

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

Remarks. The molecular study of several museum specimens from North Africa, classified as *R. brondelii* from morphology (Caldara *et al.*, 2008), confirmed that these specimens were rightly identified. Moreover, the fact that the *COII* sequence of the three specimens from Morocco corresponds with that of the specimens from Tunisia and Algeria suggests that in North Africa there is only one species of this group, and that *R. lanuginosa* is synonymous with *R. brondelii*. The host plant association of the North African populations of *R. brondelii* remains uncertain. The host plant of the specimens from Tunisia could be the widely distributed *Linaria cossoni* Bonn. & G. Barr., a toadflax species which is considered to be conspecific with *L. purpurea* from Italy and Sicily (Viano, 1978; Sutton, 1988). According to Hoffmann (1958) and data obtained from labels attached to archived specimens, *Linaria reflexa* (L.) Chaz. may be a potential host plant for North African populations, but in general further study is needed to determine host range of North African populations of this species.

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

http://zoobank.org/urn:lsid:zoobank.org:act:2D3FCB34-F2FD-4F84-9130-E165BC492CC8. (Figs 1C, 6G–I).

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

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

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

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

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

Rhinusa eversmanni inhabit galls induced by *R. rara*. The intruder oviposits in fully developed galls of *R. rara* **sp.n.** and its presence may have a strong negative impact on *R. rara* populations. Unlike for galls induced by *R. pilosa* on *L. vulgaris*, where emergence of both weevil species is synchronized and

phenology partly overlaps, the attack rate by *R. eversmanni* on *R. rara* galls strongly depends on temperature fluctuation during the early spring. The negative impact of the gall intruder may increase after a prolonged period of low temperature during the early spring. The interaction between *R. rara* and its intruder is very important and may perhaps result in local extinction of the gall inducer. To date, all known habitats with significant populations of *R. rara* are calcareous xerothermic slopes of south-east exposition. On such habitats, high temperature gradients during early spring favour rapid development of *R. rara* galls and larvae. Thus, because of early oviposition behaviour and rapid larval development inside induced galls, most larvae of *R. rara* are already at L3 instars when *R. eversmanni* oviposition begins.

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

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

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

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

Discussion

The study of *R. pilosa* was primarily undertaken because of its potential use as a biological control agent for invasive toadflaxes in North America. The taxonomic focus in the early stages of the biological control programme was attributed to be *R. hispida* (Brullé, 1832), following literature references describing this taxon as a 'well known' gall inducer on *L. vulgaris*. In fact, the identity of this taxon had been uncontroversial for more than one century because of its easy differentiation from all other species of *Rhinusa* by a markedly pilose body (Desbrochers des Loges, 1893; Reitter, 1907, 1916; Hustache, 1931; Hoffmann, 1958; Smreczyński, 1976; Lohse & Tischler, 1983). However, after the examination of a type specimen, Caldara *et al.* (2008) established that *R. hispida sensu auctorum* is not the same species as *R. hispida* (Brullé, 1832), which is actually a synonym of *Rhinusa tetra* (Fabricius, 1792). In addition, Caldara *et al.* (2008) stated that under the name *R. hispida sensu auctorum* two distinct taxa were confused, *R. pilosa* (Gyllenhal, 1838) and *R. brondelii* (Brisout de Barneville, 1862), which are distinguishable from each other by taxonomic, 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 south-eastern 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 programmes 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 programmes 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 relation 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 & Shorthouse, 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). By 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 and specific events followed by the production of new vascular tissue and the occurrence of massive cellular hyperplasy and hypertrophy (Barnewall & De Clerck-Floate, 2012).

The complexity and specificity among gall inducers with regard to host plant use and specific plant organs galled are substantial and well documented (Manni, 1964; Floate *et al.*,

1996; Abrahamson *et al.*, 1998). In our gall-inducing and host suitability study tests, oviposition of *R. pilosa* on *L. genistifolia* resulted in high number of hypersensitive reactions which practically blocked gall development, and thus any possibility of larval development. On rare occasions, we have registered both gall and larval development, but only on *L. genistifolia* plants in very early phenophase of shoot growth.

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

On the basis of the genetic data, the *R. pilosa* complex forms two subgroups, as considered by Caldara *et al.* (2010), that is, the *R. pilosa* and *R. brondelii* subgroups. When applying a mean substitution rate of 0.0152 subs/s/myr, the timescale of divergence among the main lineages of the *R. pilosa* species complex seems consistent with geological events in southern Europe and North Africa (Table 6). These two subgroups (Fig. 3, node II) are estimated to have diverged from each other approximately 7.2 mya (95% HPD, 4.2–11.1), placing their initial divergence time within a period of dramatic geological events in southern Europe during the late Miocene (11.1–5.3 mya). The estimated divergence times of 4.7 mya (95% HPD, 2.5–7.7) associated with subsequent speciation within the *R. brondelii* subgroup (node III) coincides with the period after the Messinian salinity crisis, dated between 5.6 and 5.3 mya (Krijgsman *et al.*, 1999), when a broad range of new land connections existed (De Jong, 1998) and the dispersal of floristic and faunistic elements across the Mediterranean basin (Montreuil, 2008) was possible, prior to rapid inundation during the Zanclean flood (Garcia-Castellanos *et al.*, 2009). Consistent with these events, our results indicate the possibility of biogeographical vicariance for the origin of the two lineages within the *R. brondelii* subgroup. However, with no phylogenetic framework for the genus *Linaria*, it is not possible to evaluate if the different host associations of the two lineages were subsequently forced by vicariant host–plant association or were coincident with it, both of which could explain *R. brondelii* associated with *L. purpurea* (southern Italy and North Africa) and *R. rara* associated with *L. genistifolia/dalmatica* (south-east Europe). A similar biogeographical scenario of vicariance in the Mediterranean Basin has been documented for Pachydeminae beetles (Sanmartín, 2003), and also in the Mecinini weevils from the *Mecinus heydenii* species complex (Toševski *et al.*, 2014).

The taxonomic verification of the archival material of *R. brondelii* with SPIAs reveals the unity of Calabrian, Sicilian and North African specimens of this species. If we consider the opinion of Viano (1978) about the conspecific status of the North African *Linaria cossoni* with regard to the Italian *L. purpurea*, then the link between Tunisian, Algerian and Sicilian *R. brondelii* becomes more than obvious. There are many documented studies that support intensive transitions of biota during major glaciation events during the Pleistocene and that can explain the origin of vicariant populations distributed in southern Italy and North Africa (Sanmartín, 2003; Maryańska-Nadachowska *et al.*, 2010; Condamine *et al.*, 2013).

The evolutionary history of *R. pilosa* seems to be less than simple. No differences in morphology were found among studied populations, or in their biology or phenology. For all studied populations, *L. vulgaris* was found to be the host plant. By contrast, substantial divergence at genetic level was found, with two mtDNA lineages, Rp-A and Rp-B, with estimated divergences of the mitochondrial markers *COII* and *16S* of 4% and 1.6% respectively. Several authors (e.g. DeSalle *et al.*, 1987; Brower, 1994) have proposed that divergence of mitochondrial markers in excess of 2%, or even less (Hebert *et al.*, 2004), may correlate with the existence of cryptic species. In our molecular study we have excluded the presence of nuclear paralogues as a possible explanation of the high mtDNA sequence divergences observed. On the other hand, sequencing of nucler *EF-1α* gene revealed only three haplotypes within *R. pilosa* populations, two of them (EF-Rp1 and EF-Rp3) common for both mitochondrial lineages Rp-A and Rp-B. Thus, our analyses revealed no association between the distribution of mitochondrial haplotype groups and either geography or nuclear genomic variation, which, combined with ecological and morphological factors, argues against the possibility that the different mtDNA haplotype groups may reflect the early stages of speciation, or cryptic speciation. An additional important piece of evidence against cryptic species within *R. pilosa* is the frequent appearance of both mtDNA lineages in syntopy, while biological tests revealed no signs of reproductive isolation associated with the two divergent mtDNA lineages (I. Toševski, unpublished data).

Deep intraspecific mitochondrial divergence could be correlated with *Wolbachia* infection (Smith *et al.*, 2012; Xiao *et al.* 2012; Zahiri *et al.*, 2014), even if the pathway of this mechanism is still not clear (Werren *et al.*, 2008). We detected *Wolbachia* infection within populations of both *R. pilosa* and *R. rara* **sp.n.** However, our data strongly suggest that mitochondrial divergence between Rp-A and Rp-B mitochondrial haplotype groups within *R. pilosa* is not impacted by *Wolbachia* presence, because the common *Wolbachia* allele WRp was recorded in all 14 haplotypes belonging to both mitochondrial groups. This excludes a *Wolbachia* 'sweeping effect' on haplotype diversity through cytoplasmatic incompatibility, while it seems more obvious that abundance of haplotypes is likely driven by a metapopulation effect or, possibly but less probably, the adaptive ability of particular haplotypes *in situ*. Moreover, we have observed equal and balanced sex ratios inside *R. pilosa* populations during mass rearing experiments with field-collected *R. pilosa* that were part of a biological control programme between 2004 and 2012

(I. Toševski, unpublished data). This excludes the three other distinct reproductive phenotypes caused by *Wolbachia,* i.e. male killing, feminization and parthenogenesis. Nevertheless, it is obvious that populations of *R. pilosa* consist of deeply divergent mitochondrial haplotype groups, with unusually high divergences between haplotypes within groups (e.g. Rp-B). Based on the evidence, the most plausible explanation is that the divergence of *R. pilosa* mitochondrial lineages evolved in allopatry, with subsequent admixture proceeding from secondary contact between previously separated populations (Futuyma, 2005).

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

Our molecular data suggest that within glacial and inter-glacial cycles during the Pleistocene, populations of *R. pilosa* probably experienced successive periods of isolation that led to 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 agreement with Coope (1994) and Abellán *et al.* (2011), who argue for intensive range changes of insects during the Quaternary by comparing fossil records and contemporary distributions.

The validation of taxon boundaries, especially inside groups burdened with taxonomic uncertainty, is one of the more important and fundamental issues within biological control programmes. 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 to this species complex; however, increased taxon and molecular sampling across the western Palearctic is required for a proper evaluation.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12109

Appendix S1. List of specimens collected for morphological and molecular analyses, sorted by species name, accession number of *COII* gene deposited in NCBI GenBank, host plant affiliation, locality, voucher and haplotype name.

Appendix S2. Archival specimens from MNHN Paris collection for DNA analysis.

Appendix S3. List of specimens used in this study, sorted by species name, accession number of *16S* rRNA gene deposed in NCBI GenBank, host plant affiliation, locality, voucher and haplotype name.

Appendix S4. Primer design and additional PCR protocols for confirmation of orthologous character of *COII* gene sequence.

Appendix S5. List of specimens used for the study of the elongation factor-1α gene, sorted by accession number of sequences deposited in NCBI GeneBank, host plant affiliation, locality, GPS coordinate, voucher code, *COII* haplotype and *EF-1*α haplotype name.

Appendix S6. Concatenated short phylogenetically informative sequences of *COII* gene derived from archival specimens deposited in the MNHN Paris collection.

Appendix S7. List of specimens for *Wolbachia* study, sorted by accession number of *coxA* gene sequences deposited in NCBI GeneBank, host plant affiliation, locality, GPS coordinate, sex, voucher code, *COII* genotype and *Wolbachia* strain name.

Appendix S8. Gall induction patterns of the species from the *Rhinusa pilosa* species complex, with regard to their authentic host plants from the genus *Linaria*.

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Appendix S1.

Material examined:

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

SERBIA: Dobanovci, ex l., ex *L. vulgaris*, N44 50.332 E20 09.396, 72 m, 10.07.2008, lgt. I.Toševski (8♂, 10♀, ITC); same locality, ex l., ex *L. vulgaris*, 15.07.2010 (50♂, 50♀ ITC; 50♂, 50♀, RCCM; 25♂, 25♀ CABIC; 6♂, 6♀ MNHN ; 6♂, 6♀, BMNH); Petrovo Selo, Kladovo, ex l., ex *L. vulgaris,* N44 37.632 E22 31.667, 158 m, 31.05.2008, lgt. I.Toševski (3♂, 2♀, ITC); Srednjevo, Požarevac, ex l., ex *L. vulgari,* N44 39.030 E21 28.355, 162 m, 15.07.2006, lgt. I.Toševski 2♀, ITC); Umčari, ex l., ex *L. vulgaris,* N44 34.741 E20 47.350, 146 m, 15.07.2006, lgt. I.Toševski (3♂, 2♀, ITC); Zaječar, ex l., ex *L. vulgaris,* N44 00.352 E22 18.216, 255 m, 15.07.2006, lgt. I.Toševski (1♂, ITC); Zemun, ex l., ex *L. vulgaris,* N44 50.419 E20 21.125, 84 m, 15.07.2006, lgt. I.Toševski 2♀, ITC); HUNGARY: Simontornya, ex l., ex *L. vulgaris,* N46 45.741 E18 31.509, 115 m, 2.05.2010, lgt. I.Toševski (3♂, 2♀, ITC); ROMANIA: Popricani, **Iaşi,** ex l., ex *L. vulgaris,* N47 16.414 E27 29.839, 93 m, 29.06.2010, lgt. I.Toševski (12♂, 17♀, ITC); same locality, ex l., ex *L. vulgaris*, 15.07.2011 (2♂, 2♀ ITC).

***Rhinusa brondelii* (Brisout de Barneville, 1862)**

ALGERIA: Bône, II.1859 (1, MNHN); Bou Saada (1, MNHN); Alger, Maison Carrée (3, MNHN); St. Charles, leg. Théry (2, MNHN). TUNISIA: Kairouan (1, MNHN); Souk-el-Arba, leg. Normand (6, MNHN; 4, MSNG; 1, MSNM); Sousse, Chott Mariem, ex gall on stem of *L. reflexa*, V.1984 (6, MNHN); Tunis, leg. Reitter (1, SMTD). MAROCCO: Cap Cantin near Safi (2, MNHN); Korifla, 12.V.1932, leg. Bremond (1, BMNH); Rabat (1, MNHN). ITALY: Tuscany, Talamone (Grosseto), 20.III.1993, leg. Bellò (1, GOCA); Abruzzo, Montagne Morrone Bagnaturo (L'Aquila), Colle Vacche, 1174–1552 m, 27.VII.1997, ex gall, ex *L. purpurea*, leg. Presutto (2, GOCA); Sicily, Catania, on *L. purpurea*, leg. Sacca (1, LMCP); Calabria, Montalto, Aspromonte Massif, N38 08.166 E 15 51.243, 1565 m, 22.VII.2011, ex gall, ex *L. purpurea*, leg. Baviera (ITCB 20, RCCM 20, CBCM 20); ; Sicilia, Catania, Mount Etna, Monte Nero, N37 47.857 E15 01.640, 2040 m, 15.07.2011, ex gall, ex *L. purpurea*, leg. Baviera (ITCB 20, RCCM 10, CBCM 10).

List of specimens used to extract DNA in this study, sorted by species name, accession number of COII gene deposited in NCBI GeneBank, host plant affiliation, locality, voucher and haplotype name.

DNA accession code	Host plant	Location / country	GPS coordinates	voucher and sequence code	Haplotype name
***Rhinusa pilosa,* COII haplotype group Rp-A**					
	Linaria vulgaris	Zemun Serbia	N44 50.419 E20 21.125, 84 m	SRB-MM8	
	Linaria vulgaris	Dobanovci Serbia	N44 50.332 E20 09.396, 72 m	SRB-335	
	Linaria vulgaris	Dobanovci Serbia	N44 50.332 E20 09.396, 72 m	SRB-2195	
	Linaria vulgaris	Dobanovci Serbia	N44 50.332 E20 09.396, 72 m	SRB-2860	
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	SRB-2196	
	Linaria vulgaris	Petrovo Selo, Serbia	N44 37.632 E22 31.667, 158 m	SRB-2198	
	Linaria vulgaris	Petrovo Selo, Serbia	N44 37.632 E22 31.667, 158 m	SRB-2868	
	Linaria vulgaris	Petrovo Selo, Serbia	N44 37.632 E22 31.667, 158 m	SRB-2869	
	Linaria vulgaris	Zajecar Serbia	N44 00.352 E22 18.216, 255 m	SRB-MM2	
	Linaria vulgaris	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	H-998	
KJ420489	*Linaria vulgaris*	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	H-999	**Rp-A1**
	Linaria vulgaris	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	H-1001	
	Linaria vulgaris	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	H-2888	
	Linaria vulgaris	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	H-2889	
	Linaria vulgaris	Umcari, Serbia	N44 34.741 E20 47.350, 146 m	SRB-2862	
	Linaria vulgaris	Petrovo selo, Serbia	N44 37.632 E22 31.667, 158 m	SRB-2863	
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	SRB-2919	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2899	
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	SRB-2942	
	Linaria vulgaris	Dobanovci Serbia	N44 50.332 E20 09.396, 72 m	SRB-2920	
	Linaria vulgaris	Dobanovci Serbia	N44 50.332 E20 09.396, 72 m	SRB-2922	
	Linaria vulgaris	Dobanovci Serbia	N44 50.332 E20 09.396, 72 m	SRB-2923	
KJ420490	*Linaria vulgaris*	Zemun, Serbia	N44 39.030 E21 28.355, 162 m	SRB-MM3	**Rp-A2**
	Linaria vulgaris	Zemun, Serbia	N44 39.030 E21 28.355, 162 m	SRB-2861	
KJ420491	*Linaria vulgaris*	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	SRB-MM4	**Rp-A3**
KJ420492	*Linaria vulgaris*	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	SRB-MM5	**Rp-A4**
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	SRB-MM7	
KJ420493	*Linaria vulgaris*	Petrovo Selo Serbia	N44 37.632 E22 31.667, 158 m	SRB-337	**Rp-A5**

Rhinusa pilosa, COII haplotype group Rp-B

	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-MM1	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-MM6	
KJ420494	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1005	**Rp-B1**
	Linaria vulgaris	Dobanovci Serbia	N44 50.332 E20 09.396, 72 m	SRB-2946	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1602	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2960	
	Linaria vulgaris	Popricani, Iaşi, Romania	N46 45.741 E18 31.509, 115 m	ROM-1002	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1604	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2896	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2898	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2897	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2900	
KJ420495	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2901	**Rp-B2**
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2902	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2926	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2927	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2928	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2956	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2961	
KJ420496	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1004	**Rp-B3**
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1603	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1606	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2924	
KJ420497	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2925	**Rp-B4**
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2881	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2959	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2962	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1607	
KJ420498	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2885	**Rp-B5**
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2886	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2887	
	Linaria vulgaris	Petrovo Selo, Serbia	N44 37.632 E22 31.667, 158 m	SRB-2197	
KJ420499	*Linaria vulgaris*	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2953	**Rp-B6**
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2954	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2199	
	Linaria vulgaris	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	SRB-2867	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2921	
KJ420500	*Linaria vulgaris*	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	SRB-2948	**Rp-B7**
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2949	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2950	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2952	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2870	
KJ420501	*Linaria vulgaris*	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2871	**Rp-B8**
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2873	

KJ420502	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2957	**Rp-B9**

Rhinusa pilosa, COII pseudogenes

KJ420510	*Linaria vulgaris*	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2871(Rp-B8)	**Psg1**
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2896 (Rp-B2)	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1603 (Rp-B4)	
	Linaria vulgaris	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	SRB-MM4 (Rp-A3)	
	Linaria vulgaris	Petrovo Selo, Serbia	N44 37.632 E22 31.667, 158 m	SRB-2197 (Rp-B6)	
	Linaria vulgaris	Dobanovci Serbia	N44 50.332 E20 09.396, 72 m	SRB-2873 (Rp-B8)	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1004 (Rp-B3)	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2957 (Rp-B9)	
	Linaria vulgaris	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	SRB-2867 (Rp-B7)	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-MM1 (Rp-B1)	
	Linaria vulgaris	Simontornya, Hungary	N46 45.741 E18 31.509, 115 m	H-2888 (Rp-A1)	
	Linaria vulgaris	Zemun, Serbia	N44 39.030 E21 28.355, 162 m	SRB-MM3 (Rp-A2)	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2900 (Rp-B2)	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2199 (Rp-B7)	
KJ420511	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2885 (Rp-B5)	**Psg2**
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2886 (Rp-B5)	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2887 (Rp-B5)	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2902 (Rp-B5)	
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	SRB-MM7 (Rp-B4)	

DNA accession code	Host plant	Location / country	GPS coordinates	voucher and sequence code	Haplotype name

Rhinusa brondelii, COII haplotypes group

KJ420507	*L. purpurea*	Montalto, Aspromonte, Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	CAL-2934	**bron1**
	L. purpurea	Montalto, Aspromonte, Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	CAL-2936	
	L. purpurea	Montalto, Aspromonte, Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	CAL-2937	
	L. purpurea	Montalto, Aspromonte, Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	CAL-3220	
	L. purpurea	Montalto, Aspromonte, Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	CAL-3222	
KJ420508	*L. purpurea*	Mt. Etna, Monte Nero, Sicily	N37 47.857 E15 01.640, 2040 m	SIC-1478	**bron2**
	L. purpurea	Mt. Etna, Monte Nero, Sicily	N37 47.857 E15 01.640, 2040 m	SIC-1479	
	L. purpurea	Mt. Etna, Provenzana, Sicily	N37 47.799 E15 02.242, 1800 m	SIC-2935	
	L. purpurea	Mt. Etna, Provenzana, Sicily	N37 47.799 E15 02.242, 1800 m	SIC-2938	
KJ420509	*L. purpurea*	Mt. Etna, Provenzana, Sicily	N37 47.799 E15 02.242, 1800 m	SIC-1476	**bron3**
	L. purpurea	Mt. Etna, Provenzana, Sicily	N37 47.799 E15 02.242, 1800 m	SIC-1477	

DNA accession code	Host plant	Location / country	GPS coordinates	voucher and sequence code	Haplotype name
***Rhinusa rara* sp. n., COII haplotypes group**					
	Linaria dalmatica	**Staničenje, Pirot, Serbia (Holotype)**	**N43 12.915 E22 30.495, 364 m**	**SRB-350***	
	Linaria dalmatica	Staničenje, Pirot, Serbia	N43 12.915 E22 30.495, 364 m	SRB-2929*	
	Linaria genistifolia	Aleksinac, Niš, Serbia	N43 34.495 E21 40.340, 185 m	SRB-2932*	
	Linaria genistifolia	Aleksinac, Niš, Serbia	N43 34.495 E21 40.340, 185 m	SRB-2933*	
	Linaria genistifolia	Sićevo, Niš, Serbia	N43 18.698 E22 01.990, 232 m	SRB-351*	
	Linaria genistifolia	Aleksinac, Nis, Serbia	N43 34.495 E21 40.340, 185 m	SRB-PB3*	
KJ420503	*Linaria dalmatica*	Staničenje, Pirot, Serbia	N43 13.080 E22 30.218, 379 m	SRB-1594*	**rara1**
	Linaria genistifolia	Aleksinac, Nis, Serbia	N43 34.495 E21 40.340, 185 m	SRB-1595*	
	Linaria dalmatica	Staničenje, Pirot, Serbia	N43 13.080 E22 30.218, 379 m	SRB-1596*	
	Linaria dalmatica	Staničenje, Pirot, Serbia ot	N43 13.080 E22 30.218, 379 m	SRB-1597*	
	Linaria genistifolia	Aleksinac, Nis, Serbia	N43 34.495 E21 40.340, 185 m	SRB-1599*	
	Linaria genistifolia	Aleksinac, Nis, Serbia	N43 34.495 E21 40.340, 185 m	SRB-1600*	
	Linaria genistifolia	Aleksinac, Nis, Serbia	N43 34.495 E21 40.340, 185 m	SRB-1601*	
	Linaria genistifolia	Sićevo, Niš, Serbia	N43 18.698 E22 01.990, 232 m	SRB-2891*	
KJ420504	*Linaria genistifolia*	Pirot Staničenje, Pirot, Serbia	N43 12.915 E22 30.495, 364 m	SRB-2930*	**rara2**
KJ420505	*Linaria genistifolia*	Krasnodar, Gulkewitschi, Russia	N45 17.882 E40 46.512, 121 m	RU-1354*	**rara3**
	Linaria genistifolia	Krasnodar, Gulkewitschi, Russia	N45 17.882 E40 46.512, 121 m	RU-1355*	
	Linaria genistifolia	Krasnodar, Gulkewitschi, Russia	N45 17.882 E40 46.512, 121 m	RU-1356*	
	Linaria genistifolia	Krasnodar, Gulkewitschi, Russia	N45 17.882 E40 46.512, 121 m	RU-1357*	
KJ420506	*Linaria genistifolia*	Balaton, Hungary	N46 53.142 E17 42.882, 126 m	H-2468	**rara4**
	Linaria genistifolia	Balaton, Hungary	N46 53.142 E17 42.882, 126 m	H-2469	

*specimens designated as type material.

Appendix S2.

Rhinusa brondelii Brisout, 1862
(archival specimens from MNHN Paris collection for DNA analysis)

[for details see Caldara et al. (2008) On the identity of *Rhinusa hispida* (Brullé) and its current synonyms (Coleoptera: Curculionidae), *Zootaxa*, 1805:61-68.]

No.	labels	voucher specimens
1	#Philipville (present time **Skikda)** Algérie, ex Bonnaire ; *pilosum* v. *Brondeli* -H. Bris -; #*G. hispidulum* Brull.-; #Museum Paris, 1968, Col. A.Hoffmann; #**NEOTYPE** *Gymnetron pilosum* var. *brondelii* Brisout, 1862 (des. Caldara, 2008) ; #*Rhinusa brondelii* (H.Bris.), Caldara det. 2008; #DNA IT-2532	
2	#Alger, Maison Carrée ; #*Gymnetron pilosum* Gyll. #*Gymnetron hispidulum* Brullé; #Museum Paris, 1968, Col. A.Hoffmann; #*Rhinusa brondelii* (H.Bris.), Caldara det. 2008; #DNA IT-2533	
3	#Alger, Maison Carrée ; #*Gymnetron hispidulum* Brullé; #Museum Paris, 1968, Col. A.Hoffmann; #*Rhinusa brondelii* (H.Bris.), Caldara det. 2008; #DNA IT-2534	

No.	labels	voucher specimens
4	#Alger, Maison Carrée ; #G. hispidulum Brullé; #Museum Paris, 1968, Col. A.Hoffmann; # *Rhinusa brondelii* (H.Bris.), Toševski det. 2013; #DNA IT-2535	
5	#Tunisie, Souk-el-Arba, Dr Normand; #Rhinusa brondelii (H.Bris.), Caldara det. 2008; #Museum Paris, 1968, Col. A.Hoffmann; # *Rhinusa brondelii* (H.Bris.), Toševski det. 2013; #DNA IT-2536	
6	#Tunisie, Souk-el-Arba, Dr Normand; #*Rhinusa brondelii* (H.Bris.), Caldara det. 2008; #Museum Paris, 1968, Col. A.Hoffmann; #DNA IT-2537	

No.	labels	voucher specimens
7	#Slovakia mer., Štúrovo, Fremuth, 1.VI.1960; #G. hispidum Brulle, Fremuth det. 1975; #Museum Paris 1984 coll. G. Tempere; #*Rhinusa brondelii* (H. Bris.) Caldara det. 2008; #**PARATYPE**, *Rhinusa rara* sp. n., Toševski & Caldara, 2013 #DNA-IT 2538	
8	#Tunisie Souk-el-Arba, Dr Normand; #Museum Paris 1984 coll. G. Tempere; #*Rhinusa brondelii* (H. Bris.) Caldara det. 2008; #DNA IT-2539	
9	#Kairouan; #*G. pilosum*; #Museum Paris 1980 coll. G. Ruter; #*Rhinusa brondelii* (H. Bris.) Caldara det. 2008; #DNA IT-2540.	

No.	labels	voucher specimens
10	#Rabat, #*Gymnetron hispidulum*; #Museum Paris 1980 coll. G. Ruter; #*Rhinusa brondelii* (H. Bris.) Caldara det. 2008; #DNA IT-2541.	
11	#Cap Cantin, pr. Safi, Maroc; #*Gymnetron hispidulum* v. *brondeli*; #Museum Paris 1980 coll. G. Ruter; # *Rhinusa brondelii* (H. Bris.) Caldara det. 2008; #DNA IT-2542	
12	#Cap Cantin, pr. Safi, Maroc; #Museum Paris 1980 coll. G. Ruter; # *Rhinusa brondelii* (H. Bris.) Caldara det. 2008; #DNA IT-2543	

No.	labels	voucher specimens
13	#Souk-el-Arba, Tunise. Dr. Norm. #Museum Paris 1980 coll. G. Ruter; #*Rhinusa brondelii* (H. Bris.) Caldara det. 2008; #DNA IT-2544	
14	#Souk-el-Arba, #Museum Paris 1980 coll. G. Ruter; #*Rhinusa brondelii* (H. Bris.) Caldara det. 2008; #DNA IT-2545	
15	#Souk-el-Arba, #Museum Paris 1980 coll. G. Ruter; #*Rhinusa brondelii* (H. Bris.) Caldara det. 2008; #DNA IT-2546	

#Philipville (present time **Skikda)** Algérie, ex Bonnaire ;
pilosum v. *Brondeli* -H. Bris -;
#*G. hispidulum* Brull.-;
#Museum Paris, 1968, Col. A.Hoffmann;
#**NEOTYPE** *Gymnetron pilosum* var*. brondelii* Brisout, 1862 (des. Caldara, 2008) ;
#*Rhinusa brondelii* (H.Bris.), Caldara det. 2008;
#DNA IT-2532

#Slovakia mer., Štúrovo, Fremuth, 1.VI.1960;
#*G. hispidum* Brulle, Fremuth det. 1975;
#Museum Paris 1984 coll. G. Tempere;
#*Rhinusa brondelii* (H. Bris.) Caldara det. 2008;
#**PARATYPE**, *Rhinusa rara* sp. n., Toševski & Caldara, 2014
#DNA-IT 2538

Appendix S3. List of specimens used in this study, sorted by species name, accession number of 16S rRNA gene deposited in NCBI GeneBank, host plant affiliation, locality, voucher and haplotype name.

Accession number	Host plant	Location/country	GPS coordinates	voucher and sequence code	Haplotype name
***Rhinusa pilosa,* mt16S rRNA haplotype group 16S-A**					
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2860	
	Linaria vulgaris	Zemun, Serbia	N44 39.030 E21 28.355, 162 m	SRB-2861	
KJ420513	*Linaria vulgaris*	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	SRB-MM4	**16S-A1**
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	SRB-MM5	
	Linaria vulgaris	Petrovo Selo Serbia	N44 37.632 E22 31.667, 158 m	SRB-337	
***Rhinusa pilosa,* mt16S rRNA haplotypes group 16S-B**					
KJ420514	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2960	**16S-B1**
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2946	
KJ420515	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2901	**16S-B2**
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2924	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2885	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2957	
KJ420516	*Linaria vulgaris*	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1004	**16S-B3**
KJ420517	*Linaria vulgaris*	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2953	**16S-B4**
KJ420518	*Linaria vulgaris*	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2952	**16S-B5**
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2873	
***Rhinusa brondelii,* mt16S rRNA haplotype group**					
KJ420522	*L. purpurea*	Montalto, Aspromonte, Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	CAL-2934	**16S-bron1**
	L. purpurea	Mt. Etna, Provenzana, Sicily	N37 47.799 E15 02.242, 1800 m	SIC-2935	
KJ420523	*L. purpurea*	Mt. Etna, Provenzana, Sicily	N37 47.799 E15 02.242, 1800 m	SIC-1476	**16S-bron2**
***Rhinusa rara* sp.n., mt16S rRNA haplotypes group**					
KJ420519	*Linaria dalmatica*	Staničenje, Pirot, Serbia	N43 12.915 E22 30.495, 364 m	SRB-2929	**16S-rara1**
	Linaria genistifolia	Pirot Staničenje, Pirot, Serbia	N43 12.915 E22 30.495, 364 m	SRB-2930	
KJ420520	*Linaria genistifolia*	Krasnodar, Gulkevichi, Russia	N45 17.882 E40 46.512, 121 m	RU-1354	**16S-rara2**
KJ420521	*Linaria genistifolia*	Balaton, Hungary	N46 53.142 E17 42.882, 126 m	H-2468	**16S-rara3**
***Rhinusa melas,* mt16S rRNA (outgroup)**					
KJ420512	*Chenorhinum minus*	Mokra Gora, Serbia	N43 49.380 E19 31.045, 871 m	SIC-2934	**16S-melas**

Appendix S4.

Primers design and additional PCR protocols for confirmation of orthologous character of mtCOII gene sequence

The sequences of primers used for additional sequencing of COII gene are shown in Table S4-1. As a matrix for the design of new primers, we aligned sequences amplified by primers HCO2198f/TK-N-3782 that correspond to Rp-A and Rp-B genotypes of *Rhinusa pilosa*. Because sequences of the Rp-A genotype showed less ingroup divergence, we presume they could correspond to orthologous COII gene, with Rp-B representing the probable nuclear paralog (numt) of the COII gene. Under this asumption we designed primer RpAf1 (positioned at the 3'-end of COI gene) to be specific for the selective amplification of Rp-A haplotypes, following specific single nucleotide informative positions (SNIP-s) related with this genotype. In combination with TK-N-3782 primer, RpAf1 amplified a product of 838 bp in length, representing the complete mtCOII gene. PCR conditions were similar to those described for amplification with TL2-J-3038/TK-N-3782 primers, except for annealing temperature which was increased to 58°C to ensure more specific conditions, and PCR products were sequenced using the forward primer RpAf1.

For specimens belonging to Rp-A haplotype group, the sequenced product of RpAf1/TK-N-3782 confirmed full homology with previosly obtained COII sequences (TL2-J-3038/TK-N-3782 product). In contrast, sequenced products obtained from the Rp-B haplotype group were divergent from those previously obtained (TL2-J-3038/TK-N-3782 product) and represented COII nuclear paralogs of mtDNA (NUMTs), containing one or two stop codons within protein coding sequence. This third group of sequences is

here referred to as Psg group and is available from GenBank under accession numbers KJ420510 and KJ420511. The Psg paralogous sequences expressed divergences of about 10% from both Rp-A and Rp-B genotypes. Because primer pair RpAf1/TK-N-3782 generated mtCOII paralogous only from samples with a Rp-B genotype, we designed a further primer pair in an attempt to amplify only paralogous nuclear COII sequences of samples from both the Rp-A and Rp-B lineages. The newly designed primer pair Psg-Fi/Psg-R1 successfully amplified a product of 599 bp (sequenced with reverse primer) from specimens sampled from both the Rp-A and the Rp-B lineages. The paralogous mtCOII sequences obtained from representative haplotypes using primers Psg-Fi/Psg-R1 were almost identical among each other, differing only in one nucleotide substitution. When employed in phylogenetic analyses, COII paralogous sequences formed solid separate basal cluster in regard to orthologous sequences of genuine mtCOII gene obtained with TL2-J-3038/TK-N-3782 primers (Fig. S4-1).

Table S4-1. Primer pairs used for amplification of orthologous and paralogous COII mitochondrial regions in *Rhinusa pilosa* complex.

primer pairs	primer position	primer sequence (5'… 3')	Amplicon length[a] (bp)	PCR protocol	sequencing primer	origin of sequenced product
TL2-J-3038 ND3-R	tRNA-Lys tRNA-Gly	TAATATGGCAGATTAGTGCATTGGA GATTGGAAGTCAAATATAA	≈2500	direct	TL2-J-3038	orthologous
HCO2198f AspRpR*	COI gene tRNA-Asp	TGATTTTTTGGTCAGCCTGAAGTTTA GCTTGACAAGCTAATGTTATAG	1704	direct	-	orthologous
HCO2198f TK-N 3782	COI gene tRNA-Lys	TGATTTTTTGGTCAGCCTGAAGTTTA GAGACCATTACTTGCTTTCAGTCATCT	1628	direct	HCO2198f TK-N 3782	orthologous
TL2-J-3038 TK-N 3782	tRNA-Leu tRNA-Lys	TAATATGGCAGATTAGTGCATTGGA GAGACCATTACTTGCTTTCAGTCATCT	788	direct & nested	TL2-J-3038	orthologous
RpAf1* TK-N 3782	COI gene tRNA-Lys	GCACAGTCATAGGTACAGGGAA GAGACCATTACTTGCTTTCAGTCATCT	838	direct	RpAf1	orthologous & paralogous
RpAf1* Psg-R1*	COI gene COII gene paralog	GCACAGTCATAGGTACAGGGAA TATTCACCACCCCAGTTAATGT	758	direct	Psg-R1	paralogous
Psg-Fi* Psg-R1*	COII gene paralog COII gene paralog	TCATGATCATACAATGATTATTT TATTCACCACCCCAGTTAATGT	599	direct	Psg-R1	paralogous

*** primers designed for this study**
[a] length without primers

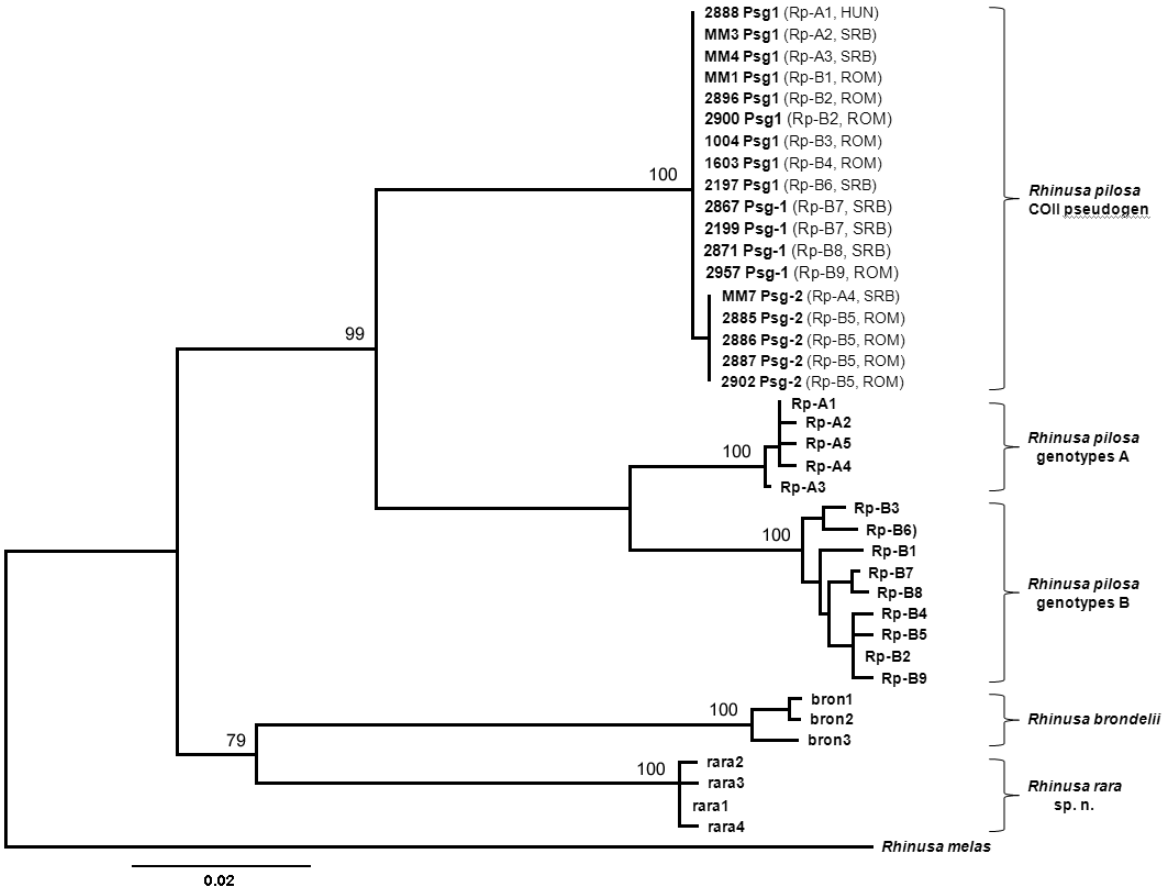

Fig. S4-1. Neighbour-joining tree (p-distance method) constructed in MEGA5 using 21 haplotypes of genuine mt COII gene recorded within the *R*. *pilosa* species complex and nuclear paralogous sequences (numts) identified within each population of *R. pilosa*. For each numt, a corresponding mtCOII haplotype and country of origin of the particular population of *R. pilosa* is given in parentheses. Bootstrap support values for main nodes are shown. There were a total of 554 bp in the final dataset.

Considering that orthology is of fundamental importance for phylogenetic studies, especially when cryptic speciation events are possibly involved (Song *et al.*, 2008), we further designed highly specific primers for mtDNA employing sequencing of the neighboring region which delimited mtCOII gene of *R. pilosa*. For this purpose we have sequenced products obtained with TL2-J-3038 and reverse complement of ND3 primer (Haran *et al.*, 2013) using TL2-J-3038 as sequencing primer. Obtained sequences of

regions adjacent to the COII gene allowed us to design specific primer AspRpR for *R. pilosa* at position of tRNA-Asp (Table S4-1). This primer was later used for selective amplification of highly diluted DNA (containing predominantly mtDNA copies) in nested PCR procedure.

To obtain reliable orthologous mitochondrial sequence of COII gene and avoid the numts, we have undertaken serial dilutions for PCR, in which genomic DNA was submitted to direct PCR followed by a nested PCR amplification protocol. We considered that in dilution of 1:6250, any PCR would contain less than one nuclear genome (Calvignac *et al.*, 2011). Thus, we supposed that in more extreme dilutions such as 1:12500 and 1:25000, the direct PCR will preferentially amplify mitochondrial gene, minimizing possibilities for amplification of nuclear paralogs, while nested PCR will enable sufficient amplification yield. Direct and nested PCR were performed using HCO2198f/AspRpR and TL2-J-3038/TK-N-3782 primer pairs, respectively. Specimens representing all previously recognized 14 COII haplotypes of *R. pilosa* from both haplotype groups were submitted to direct and nested PCR protocol. For direct PCR, genomic DNA was diluted at 1:50, 1:250, 1:1250, 1:6250, 1:12500 and 1:25000. Reaction mix was the same as for the amplification with TL2-J-3038/TK-N-3782 primers for both direct and nested PCR. The amplification conditions and protocol for nested PCR were the same as those described previously, but slightly modified for direct amplification (T_a=48°C, t_e= 3 min). Nested PCR products were sequenced using primer TL2-J-3038. Subsequently, sequences from each particular haplotype obtained from different dilutions were aligned to confirm congruence among them and with previously

sequenced products obtained with primers TL2-J-3038/TK-N 3782 using undiluted DNA extracts as template (see material and methods).

Direct PCR amplified more or less visible products in dilution up to 1:6250, while all nested reactions yielded PCR reach product (Fig. S4-2). Complete homology was found among all sequences from different dilutions of particular haplotypes, confirming orthologous character of the mtCOII sequences initially obtained with TL2-J-3038/TK-N 3782 primers in our study. These sequences were used for further phylogenetic analysis and estimation of divergence time between species from *R. pilosa* complex.

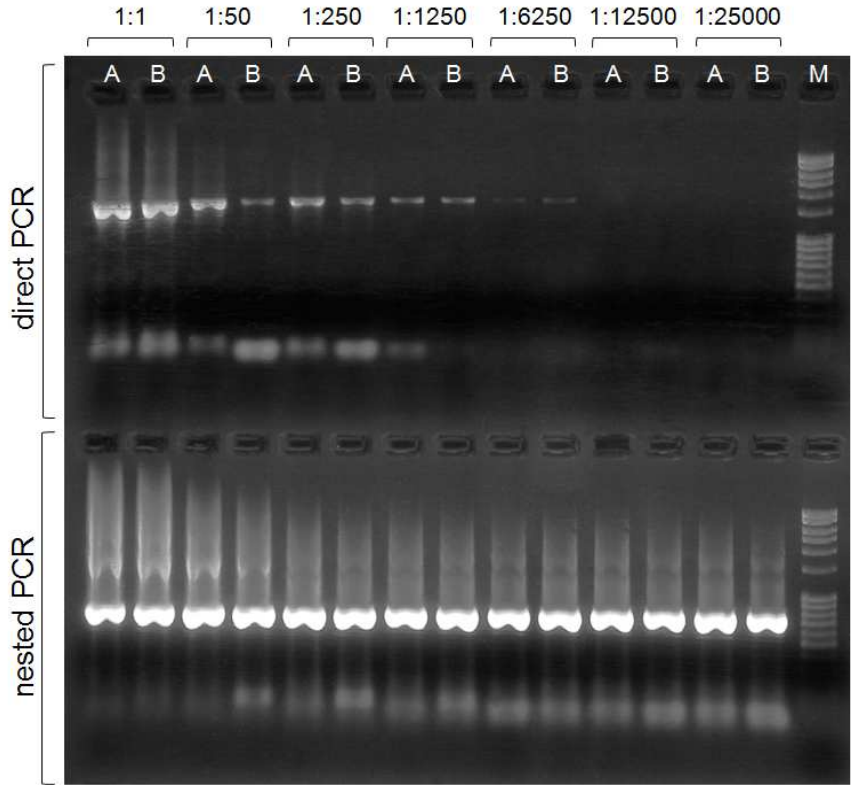

Fig S4-2. Dilution method for genuine mtDNA amplification using direct and nested PCR. Agarose gel electrophoresis of direct (HCO2198f/AspRpR) and nested (TL2-J-3038/TK-N-3782) PCR products amplified from *Rhinusa pilosa* genomic DNA extracts of specimens belonging to COII haplotype Rp-A (A) and Rp-B (B). Direct PCR amplification was performed in series of dilution from 1:50 to 1:25000 using primer pair HCO2198f and AspRpR. Nested amplifications were performed for all direct products in dilution of 1:25 using primers TL2-J-3038 and TK-N 3782.

The mitochondrial 16S ribosomal RNA gene (16S) for all *R. pilosa* COII haplotypes (see Appendix S3) was sequenced in order to compare phylogenetic relatedness using a gene positioned opposite to COII gene within the circular structure of mtDNA. For this purpose we have designed primers 16Scf1 and 16Sbr1 (see Table 4) by aligning cucujiform 16S sequences from mitochondrial genomes deposited in GenBank, which allowed us to amplify ≈1080 bp PCR product from all selected specimens from *R. pilosa* complex. Sequences of 16S gene are available from GenBank under accession numbers KJ420513- KJ420523.

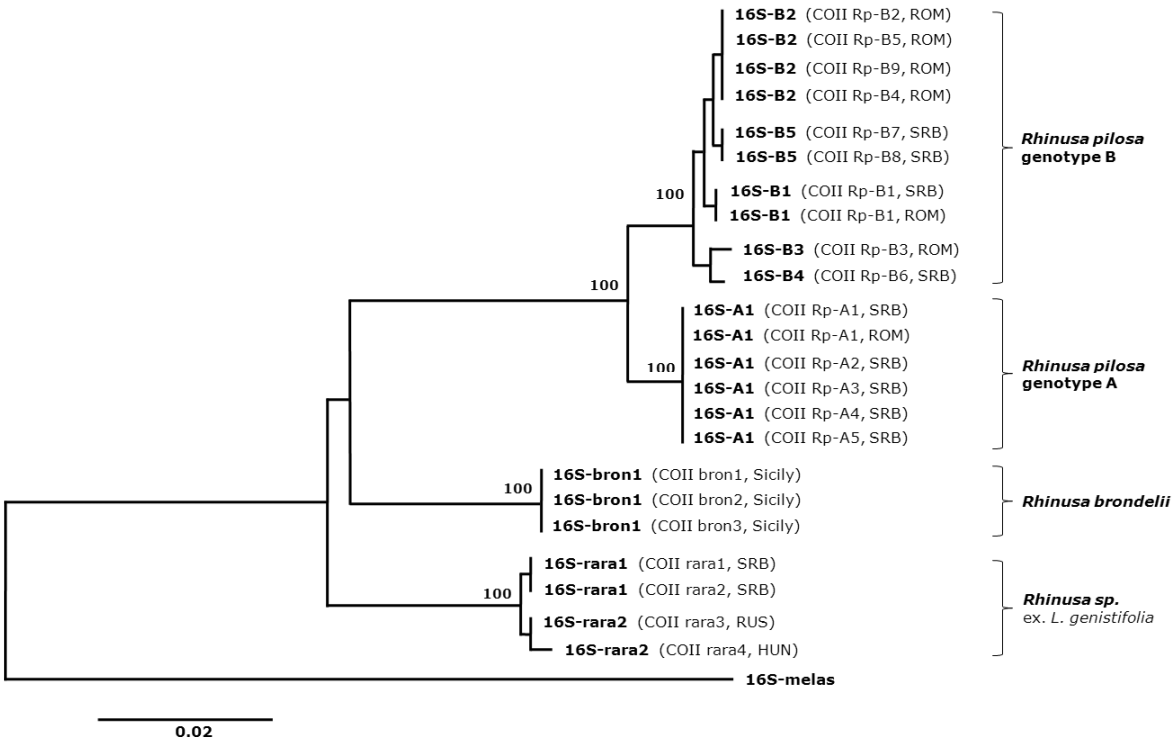

Fig S4-3. Neighbour-joining tree (p-distance) of 16S gene of *Rhinusa pilosa* species complex constructed in MEGA5, reconstructed using 21 sequences of specimens with unique mtCOII haplotype, except for Rp-A1 and Rp-B1 COII haplotypes for which two 16S sequences, each from Serbia and Romania, where applied (corresponding COII haplotypes are indicated in parentheses). Bootstrap support values for main nodes are shown. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

The final alignment of the 16S sequences consisted of a total of 951 bp, with a total of 191 (20.1%) polymorphic nucleotides of which 97 were parsimony informative. The maximum ingroup genetic distance was 7.2% (uncorrected). Neighbor-joining analysis (p-distance method) of 16S gene confirmed distinct genetic divergence associated with host plant use, and analysis was supported with highest bootstrap values.

Two mitochondrial lineages associated with *R. pilosa* were clearly divided in separate clusters (Fig S4-3), confirming authenticity of the Rp-A and Rp-B genotypes recorded on COII gene. The average genetic divergence between two *R. pilosa* genotypes 16S-A and 16-B was 1.6%, while divergence among *R. pilosa* ex *L. vulgaris, R. brondelii* ex *L. purpurea* and genotypes associated with *L. genistifolia* was 6.2-6.6% and 6.6-7.2%, respectively. *Rhinusa brondelii* diverged from specimens associated with *L. genistifolia* by 5.2% (Table S4-2).

Table S4-2. Average divergence on mitochondrial small subunit 16S ribosomal RNA based on the pairwise analysis (p-distance) among *Rhinusa pilosa* complex haplotypes, grouped according to their host plant affiliation.

Sequences group of 16S rRNA gene	d_1 (*S.E*)	d_2 (*S.E.*)	P (*S.E.*)			
			1	2	3	4
1. ex *L. vulgaris* 16S-A	0.038 (0.004)	0	-	(*0.004*)	(*0.008*)	(*0.008*)
2. ex *L. vulgaris* 16S-B		0.004 (0.001)	0.016	-	(*0.008*)	(*0.008*)
3. ex *L. purpurea*		0.002 (0.001)	0.066	0.072	-	(*0.007*)
4. ex *L. genistifolia/dalmatica*		0	0.062	0.066	0.052	-

(d_1) divergence over all sequence pairs; (d_2) divergence over sequence pairs within groups; (P) p-distance over sequence pairs between groups; (S.E.) standard error;

References

Calvignac, S., Konecny, L., Malard, F. & Douady, C.J. (2011) Preventing the pollution of mitochondrial datasets with nuclear mitochondrial paralogs (numts). *Mitochondrion*, **11**, 246–254.

Haran, J., Timmermans, M.J. & Vogler, A.P. (2013) Mitogenome sequences stabilize the phylogenetics of weevils (Curculionoidea) and establish the monophyly of larval ectophagy. *Molecular Phylogenetics and Evolution*, **67**, 156–166.

Song, H., Buhay, J.E., Whiting, M.F. & Crandall, K.A. (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences*, **105**, 3486–13491.

Appendix S5. List of specimens used for the study of elongation factor-1α gene sorted by accession number of sequences deposited in NCBI GeneBank, host plant affiliation, locality, GPS coordinate, voucher code, *COII* haplotype, and *EF-1α* haplotype name.

Accession number	Host plant	Location / country	GPS coordinates	Voucher code	COII haplotype	EF-1α haplotype name
Rhinusa pilosa						
KJ620000	*Linaria vulgaris*	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	SRB-MM4 A3	Rp-A3	**EF-Rp1**
KJ620001	*Linaria vulgaris*	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	SRB-MM7 A4	Rp-A4	**EF-Rp2**
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2960 B1	Rp-B1	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2886 B5	Rp-B5	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2953 B6	Rp-B6	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2959 B4	Rp-B4	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2961 B2	Rp-B2	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-1004 B3	Rp-B3	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	ROM-2957B9	Rp-B9	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2949 B7	Rp-B7	
	Linaria vulgaris	Petrovo Selo Serbia	N44 37.632 E22 31.667, 158 m	SRB-337 A5	Rp-A5	
KJ620002	*Linaria vulgaris*	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	SRB-2873 B8	Rp-B8	**EF-Rp3**
	Linaria vulgaris	Zajecar Serbia	N44 00.352 E22 18.216, 255 m	SRB-MM2 A1	Rp-A1	
	Linaria vulgaris	Zemun, Serbia	N44 39.030 E21 28.355, 162 m	SRB-MM3 A2	Rp-A2	
Rhinusa brondelii						
KJ620004	*Linaria purpurea*	Montalto, Aspromonte, Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	CAL-1476	bron3	**EF-bron1**
	Linaria purpurea	Mt. Etna, Monte Nero, Sicily	N37 47.857 E15 01.640, 2040 m	SIC-2934	bron1	
	Linaria purpurea	Mt. Etna, Provenzana, Sicily	N37 47.799 E15 02.242, 1800 m	SIC-2935	bron2	
***Rhinusa rara* sp.n.**						
KJ620003	*Linaria dalmatica*	Staničenje, Pirot, Serbia	N43 12.915 E22 30.495, 364 m	SRB-2929	rara1	**EF-rara1**
	Linaria genistifolia	Pirot Staničenje, Pirot, Serbia	N43 12.915 E22 30.495, 364 m	SRB-2930	rara2	
	Linaria genistifolia	Sićevo, Niš, Serbia	N43 18.698 E22 01.990, 232 m	SRB-2891	rara1	
	Linaria genistifolia	Balaton, Hungary	N46 53.142 E17 42.882, 126 m	HUN-2468	rara4	
	Linaria genistifolia	Krasnodar, Gulkevichi, Russia	N45 17.882 E40 46.512, 121 m	RU-1354	rara3	

Appendix S6.

Concatenated short phylogenetically informative sequences of COII gene derived from archival specimens deposited in the MNHN Paris collection. Each of three SPIAs containing 49, 47 and 80 bp, are demarcated with single gap.

>2532 MNHN *Rhinusa brondelii* NEOTYPE Algeria
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATCCCCTCCTTAGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGGCAGTGCTCTGAAATCTGT
GGAGCTAATCATAGATTTA

>2533 MNHN Alger
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATCCCCTCCTTAGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGGCAGTGCTCTGAAATCTGT
GGGGCTAATCATAGATTTA

>2534 MNHN Algeria
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATCCCCTCCTTAGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGGCAGTGCTCTGAAATCTGT
GGGGCTAATCATAGATTTA

>2535 MNHN Algeria
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATCCCCTCCTTAGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGACAGTGCTCTGAAATCTGC
GGAGCTAATCATAGATTTA

>**2538 MNHN Slovakia *R. rara* sp.n. PARATYPE**
AATTTACAACCCTATAATTACTGTAAAAGTAATTGGGCATCAATGATTT-
AATTGATGTAATTCACTCTTGAGCAGTCCCATCCCTAGGTGTAAAAA-
TCAAATTAATTTTAATATCAATCGAACAAGATTATTCTATGGACAATGCTCCGAAATCTGT
GGAGCCAATCATAGATTCA

>2539 MNHN Tunisia
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATCCCCTCCTTAGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGGCAGTGCTCTGAAATCTGT
GGAGCTAATCATAGATTTA

>2541 MNHN Morocco
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATCCCCTCCTTGGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGACAGTGCTCTGAAATCTGT
GGAGCTAATCATAGATTTA

>2542 MNHN Morocco
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATCCCCTCCTTGGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGACAGTGCTCTGAAATCTGT
GGAGCTAATCATAGATTTA

>2543 MNHN Morocco
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATCCCCTCCTTGGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGACAGTGCTCTGAAATCTGT
GGAGCTAATCATAGATTTA

>2544 MNHN Tunisia
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATTCCCTCCTTAGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGGCAGTGCTCTGAAATCTGT
GGAGCTAATCATAGATTTA

>2545 MNHN Tunisia
AATTTATAGACCTATAATTACTATCAAAGTAATTGGACATCAATGATTT-
AATTGATGTAATTCACTCATGAGCAATCCCCTCCTTAGGAGTAAAAA-
CCAAACTAATTTTAATATTAATCGAACAAGACTATTTTATGGGCAGTGCTCTGAAATCTGT
GGAGCTAATCATAGATTTA

>Rhinusa antirrhini, Serbia, outgroup
AGTTCATAGCCCCCTAATCACAGTAAAAGTAATCGGACACCAATGATTT-
TAGAGATGTTATCCACTCATGGGCTGTACCAAGATTAAGAATAAAAA-
TCAAACTAATTTTAATATTAATCGAACAAGTCTATTTTTTGGACAATGCTCTGAAATTTGCG
GAGCTAACCACAGATTTA

Appendix S7. List of specimens for *Wolbachia* study, sorted by accession number of *coxA* gene sequences deposited in NCBI GeneBank, host plant affiliation, locality, GPS coordinate, sex, voucher code, COII haplotype and *Wolbachia* strain name.

Accession number	Host plant	Location/country	GPS coordinates	sex	Wolbachia detection	voucher code	COII haplotype	Wolbachia strain
Rhinusa pilosa								
	Linaria vulgaris	Zajecar Serbia	N44 00.352 E22 18.216, 255 m	male	+	SRB-MM2	Rp-A1	
	Linaria vulgaris	Umcari, Serbia	N44 34.741 E20 47.350, 146 m	female	+	SRB-2862	Rp-A1	
	Linaria vulgaris	Zemun, Serbia	N44 39.030 E21 28.355, 162 m	female	+	SRB-MM3	Rp-A2	
	Linaria vulgaris	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	male	+	SRB-MM4	Rp-A3	
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	male	+	SRB-MM7	Rp-A4	
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	female	+	SRB-MM5	Rp-A4	
	Linaria vulgaris	Petrovo Selo Serbia	N44 37.632 E22 31.667, 158 m	female	+	SRB-337	Rp-A5	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-MM1	Rp-B1	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	female	+	ROM-2960	Rp-B1	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	female	+	ROM-2898	Rp-B2	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-2927	Rp-B2	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	female	+	ROM-2961	Rp-B2	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-1004	Rp-B3	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-2925	Rp-B4	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	female!	+	ROM-2959	Rp-B4	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	female	+	ROM-2885	Rp-B5	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-2886	Rp-B5	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	male	+	SRB-2953	Rp-B6	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	female	+	SRB-2949	Rp-B7	
KJ620005	*Linaria vulgaris*	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	male	+	SRB-2199	Rp-B7	**WRp**
	Linaria vulgaris	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	female	+	SRB-2867	Rp-B7	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	female	+	SRB-2873	Rp-B8	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	male	+	SRB-2195	Rp-A1	
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	male	+	SRB-2196	Rp-A1	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-2887	Rp-B5	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-2897	Rp-B2	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-2900	Rp-B2	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-2901	Rp-B2	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	+	ROM-2902	Rp-B2	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	-	ROM-2880	Rp-B2	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	male	-	ROM-2896	Rp-B2	
	Linaria vulgaris	Umčari, Serbia	N44 34.741 E20 47.350, 146 m	male	-	SRB-2942	Rp-A1	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	male	-	SRB-2943	Rp-A1	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	male	-	SRB-2944	Rp-A1	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	male	-	SRB-2945	Rp-A1	
	Linaria vulgaris	Srednjevo, Serbia	N44 39.030 E21 28.355, 162 m	male	+	SRB-2948	Rp-B7	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	male	-	SRB-2951	Rp-B7	
	Linaria vulgaris	Petrovo Selo Serbia	N44 37.632 E22 31.667, 158 m	male	-	SRB-2865	Rp-B7	
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	male	+	SRB-2871	Rp-B8	

Accession number	Host plant	Location/country	GPS coordinates	sex	Wolbachia detection	voucher code	COII haplotype	Wolbachia strain
	Linaria vulgaris	Dobanovci, Serbia	N44 50.332 E20 09.396, 72 m	male	-	SRB-2874	Rp-A1	
	Linaria vulgaris	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	(larva)	+	H-998	Rp-A1	
	Linaria vulgaris	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	(larva)	+	H-999	Rp-A1	
KJ620005	*Linaria vulgaris*	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	(larva)	+	H-1000	Rp-A1	**WRp**
	Linaria vulgaris	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	(larva)	+	H-1001	Rp-A1	
	Linaria vulgaris	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	(larva)	+	H-2888	Rp-A1	
	Linaria vulgaris	Simontornya Hungary	N46 45.741 E18 31.509, 115 m	(larva)	+	H-2889	Rp-A1	
	Linaria vulgaris	Popricani, Iaşi, Romania	N47 16.414 E27 29.839, 93 m	female	+	ROM-2957	Rp-B9	
Rhinusa brondelii								
	Linaria purpurea	Mt. Etna, Monte Nero,	N37 47.857 E15 01.640, 2040 m	female	-	SIC-2934	bron1	
	Linaria purpurea	Mt. Etna, Provenzana,	N37 47.799 E15 02.242, 1800 m	male	-	SIC-2936	bron1	
	Linaria purpurea	Mt. Etna, Provenzana,	N37 47.799 E15 02.242, 1800 m	male	-	SIC-2937	bron1	
	Linaria purpurea	Mt. Etna, Monte Nero,	N37 47.857 E15 01.640, 2040 m	female	-	SIC-1478	bron2	
	Linaria purpurea	Mt. Etna, Monte Nero,	N37 47.857 E15 01.640, 2040 m	female	-	SIC-1479	bron2	
-	*Linaria purpurea*	Mt. Etna, Provenzana,	N37 47.799 E15 02.242, 1800 m	female	-	SIC-2935	bron2	***Wolbachia*** **not detected**
	Linaria purpurea	Mt. Etna, Provenzana,	N37 47.799 E15 02.242, 1800 m	female	-	SIC-2938	bron2	
	Linaria purpurea	Montalto, , Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	male	-	CAL-1476	bron3	
	Linaria purpurea	Montalto, , Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	female	-	CAL-1477	bron3	
	Linaria purpurea	Montalto, , Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	male	-	CAL-3220	bron1	
	Linaria purpurea	Montalto, , Calabria, Italy	N38 08.166 E 15 51.243, 1565 m	male	-	CAL-3222	bron1	
Rhinusa rara **sp.n.**								
	Linaria dalmatica	Staničenje, Pirot, Serbia	N43 12.915 E22 30.495, 364 m	male	+	SRB-2929	rara1	
	Linaria genistifolia	Sićevo, Niš, Serbia	N43 18.698 E22 01.990, 232 m	female	+	SRB-2891	rara1	
	Linaria genistifolia	Pirot Staničenje, Serbia	N43 12.915 E22 30.495, 364 m	female	+	SRB-2930	rara2	
	Linaria genistifolia	Krasnodar, Russia	N45 17.882 E40 46.512, 121 m	male	-	RU-1354	rara3	
KJ620006	*Linaria genistifolia*	Sićevo, Niš, Serbia	N43 18.698 E22 01.990, 232 m	male	-	SRB-2894	rara1	**WRr**
	Linaria genistifolia	Aleksinac, Nis, Serbia	N43 34.495 E21 40.340, 185 m	male	-	SRB-1595	rara1	
	Linaria genistifolia	Staničenje, Pirot, Serbia	N43 12.915 E22 30.495, 364 m	male	-	SRB-1596	rara1	
	Linaria genistifolia	Aleksinac, Nis, Serbia	N43 34.495 E21 40.340, 185 m	male	+	SRB-1599	rara1	
	Linaria genistifolia	Aleksinac, Nis, Serbia	N43 34.495 E21 40.340, 185 m	male	+	SRB-1600	rara1	
	Linaria genistifolia	Balaton, Hungary	N46 53.142 E17 42.882, 126 m	female	+	HUN-2468	rara4	

Appendix S8.

Gall induction patterns of the species from *Rhinusa pilosa* species complex with regard to their authentic host plants from the genus *Linaria*.

A

A1) Galls induced by *Rhinusa pilosa* on *Linaria vulgaris*.

A2) Galls induced by *Rhinusa pilosa* on *Linaria vulgaris*.

B

B1) Habitat of *Rhinusa brondelii* on Mt. Etna (Sicily) with population of *Linaria purpurea*.

B2) Population of *Linaria purpurea* attacked by *Rhinusa brondelii*: Montalto, Aspromonte, Calabria,

B3) Galls induced by *R. brondelii* on *L. purpurea* (Montalto, Aspromonte, Calabria).

B4) Galls induced by *R. brondelii* on *Linaria purpurea* (Mt. Etna, Sicily).

C

C1) Habitat of *Rhinusa rara* **sp.n.** on Aleksinac (Serbia) with population of *Linaria genistifolia*.

C2) Galls induced by *Rhinusa rara* **sp.n.** on *L. genistifolia* (Aleksinac, Serbia).