Identification of Two Cryptic Species Within the *Praon abjectum* Group (Hymenoptera: Braconidae: Aphidiinae) Using Molecular Markers and Geometric Morphometrics

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ABSTRACT The genus *Praon* represents a large group of aphid endoparasitoids and is exemplary for the problems encountered in their taxonomy because of a great variability of morphological characters. To investigate the intraspecific variability and to ascertain cryptic speciation within the *Praon abjectum* Haliday group, biotypes in association with the aphid hosts *Aphis sambuci* L., *Longicaudus trirhodus* Walker, and *Rhopalosiphum* spp. were examined. We combined molecular and geometric morphometric analyses, that is, partial sequences of the mitochondrial cytochrome oxidase subunit I and nuclear 28SD2 genes and the shape of the forewing. Low variation of 28SD2 sequences confirmed the close relatedness of species from the genus *Praon*. Analysis of the cytochrome oxidase subunit I sequences however identified three separate taxa within the *P. abjectum* group with substantial genetic divergence. The biotype of *P. abjectum* associated with *L. trirhodus* differed from those associated with *Rhopalosiphum* sp. and *A. sambuci* by 5.4–6.5% and 7.7% sequence divergence, respectively, while the genetic distance between the latter two biotypes ranged from 9.5 to 10%. The main changes in the forewing shape that discriminate these three biotypes as revealed by geometric morphometrics are related to the stigma shape and the position of the radial nerve. Based on the differences determined in mitochondrial sequences and in the shape of the wing, we describe two new cryptic species within the *P. abjectum* group as follows: *P. sambuci* sp. n. in association with *A. sambuci*/*S. nigra* and *P. longicaudus* sp. n. in association with *L. trirhodus*/*T. aquilegifolium*.

KEY WORDS aphid parasitoids, host specialization, *Praon sambuci* sp.n., *Praon longicaudus* sp.n., *Longicaudus trirhodus*, *Aphis sambuci*

The genus *Praon* Haliday is one of the largest genera within the subfamily Aphidiinae (Braconidae: Hymenoptera) comprising over 50 described species (Mackauer and Starý 1967, Kavallieratos et al. 2005). They are solitary endoparasitoids of aphids (Mackauer 1959, Starý 1970) occurring throughout the Palaearctic and Nearctic, with 28 species detected in Europe (Achterberg 2011) and over 15 species in North America (Johnson 1987, Pike et al. 2000). Compared with most of the other genera from the subfamily Aphidiinae, *Praon* species express a peculiar biological trait in that larval pupation takes place mostly under the empty body of the parasitized aphid (Mackauer 1959; Starý 1970, 1981). Other aphidiine parasitoids mostly pupate inside the parasitized aphid.

Parasitoids from the genus *Praon* have an extensive and variable host range with >150 described aphid hosts, including many species that are economically important pests in different crops (Pike et al. 2000, Kavallieratos et al. 2004a, Starý 2006). Several species in the genus *Praon* were described and many new *Praon*-aphid-plant associations were also reported in Southeastern Europe over the last 15 yr (Tomanović and Kavallieratos 2002; Kavallieratos et al. 2003; Tomanović et al. 2003a,b). This genus is characteristic for many problems encountered in the taxonomy of Aphidiinae resulting from the great intraspecific variability of morphological characters commonly used in species determination (e.g., wing venation patterns, mesonotum setation) (Tremblay and Pennacchio 1985; Tomanović et al. 2003a,b). In a reconstruction of the phylogenetic relationships based on morphological characters, three species groups were recognized

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Table 1. Sampling data for specimens used in molecular analysis

Parasitoid (code)	Host aphid	Plant	Country of origin (collection site)	GenBank accession number
P. abjectum (P30)	*Rhopalosiphum* sp.	*Prunus domestica*	France (Altkrich)	KC128669 (COI) KC128680 (28S)
P. abjectum (P46)	*Rhopalosiphum* sp.	*Zea mays*	Slovenia (Strujan)	KC128670 (COI) KC128681 (28S)
P. abjectum (P59)	*Rhopalosiphum* sp.	*Prunus domestica*	Slovenia (Zalog)	KC128671 (COI) KC128682 (28S)
P. abjectum (P45_1)	*Longicaudus trirhodus*	*Thalictrum aquilegifolium*	Montenegro (Durmitor)	KC128672 (COI) KC128683 (28S)
P. abjectum (P45_2)	*Longicaudus trirhodus*	*Thalictrum aquilegifolium*	Montenegro (Durmitor)	KC128673 (COI) KC128684 (28S)
P. abjectum (P50_1)	*Aphis sambuci*	*Sambucus nigra*	Serbia (Vraćevšnica)	KC128674 (COI) KC128685 (28S)
P. abjectum (P51)	*Aphis sambuci*	*Sambucus nigra*	Serbia (Vraćevšnica)	KC128675 (COI) KC128686 (28S)
P. yomenae (P50_2)	*Uroleucon inoculata*	*Inula salicina*	Serbia (Žeželj)	KC128676 (COI) KC128687 (28S)
P. dorsale (P62)	*Corylobium avellanae*	*Corylus avellana*	Switzerland (Steinmaur)	KC128677 (COI) KC128688 (28S)
P. dorsale (P69)	*Corylobium avellanae*	*Corylus avellana*	Serbia (Golija)	KC128678 (COI) KC128689 (28S)
Areopraon silvestre[a]	*Periphyllus* sp.	*Acer pseudoplatanus*	Serbia (Kragujevac)	KC128690 (28S)
Areopraon chaitophori[b]	*Chaitophorus leucomelas*	*Populus* sp.	Serbia (Zemun)	KC128679 (COI)
Aphidius uzbekistanicus[b]	*Sitobion avenae*	*Triticum aestivum*	Germany (Ebergoetzen)	JN164741.1 (COI)

[a] Outgroup taxon used for phylogenetic analyses based on 28S D2.
[b] Outgroup taxa used for phylogenetic analyses based on COI mtDNA.

within the genus *Praon:* the "*Parapraon*," "*dorsale-yomenae*," and "*rosaecola*" groups, albeit with low bootstrap support (Kavallieratos et al. 2005, Tomanović et al. 2006).

Praon abjectum Haliday is a common parasitoid distributed throughout the Palaearctic, parasitizing mainly aphid hosts from the genera *Aphis*, *Rhopalosiphum*, and *Brachycaudus* in steppe and intermediate habitats (Kavallieratos et al. 2004b, Starý 2006). Its presence was also recorded in the Nearctic by Yu et al. (2005), still without further confirmation of this finding.

P. abjectum has been reported as a consistent member of the parasitoid guilds of aphids in cereal crops, orchards, and vegetables and black bean (Tomanović and Brajković 2001, Kavallieratos et al. 2004b, Starý 2006). Starý (2006) and Kavallieratos et al. (2004b) reviewed over 16 aphid hosts for *P. abjectum*, mainly from the genus *Aphis*, associated with over 30 cultivated and native plant species.

Starý and Němec (1986) determined a great variation of electromorphs in *P. abjectum* populations parasitizing the common elder aphid, *Aphis sambuci* L., in the urban environment. In addition, our preliminary research indicated that the biotype of *P. abjectum* associated with *Longicaudus trirhodus* Walker/*Thalictrum* spp. shows differences in several morphological traits in comparison to other biotypes.

The aim of the current study was to examine the biotypes of *P. abjectum* originating from five aphid host/plant associations using partial sequences of mitochondrial cytochrome oxidase subunit I (COI) and nuclear 28SDNA genes as well as geometric morphometric analyses of the forewing. Furthermore, we wanted to test the congruence between diversification detected by COI and 28SD2 sequence analyses and biotype separation based on morphological traits and geometric morphometrics.

Materials and Methods

Examined Material. Specimens of *P. abjectum* submitted to the analyses were collected from Serbia, Slovenia, France, Czech Republic, Switzerland, and Montenegro in association with aphids from the genera *Rhopalosiphum*, *Longicaudus*, and *Aphis* (Table 1). Plant samples bearing aphid colonies with live and mummified aphids collected in the field were transferred to a rearing cabinet and monitored for the emergence of the parasitoids.

DNA Extraction, Polymerase Chain Reaction (PCR) Amplification, and Sequencing. Material submitted to molecular analyses included specimens collected in 2011 from localities in Serbia (six specimens), Montenegro (2), France (1), Slovenia (2), and Switzerland (1). Sampling localities and data related to the specimens are summarized in Table 1. Total genomic DNA was extracted from single wasps according to a nondestructive TES method described by Mahuku (2004). Before the DNA extraction, all specimens were stored in 96% ethanol at −20°C.

The mitochondrial COI was amplified using a pair of primers LCO1490\HCO2198 (Folmer et al. 1994). Each PCR reaction was carried out in a volume of 20 μl, containing 1 μl of extracted DNA, 11.8 μl of H_2O, 2 μl of High Yield Reaction Buffer A (with 1 × Mg), 1.8 μl of $MgCl_2$ (2.25 mM), 1.2 μl of dNTP (0.6 mM), 1 μl of each primer (0.5 μM), and 0.2 μl of KAPA-*Taq*DNA polymerase (0.1U/μl) (Kapa Biosystems Inc., Boston, MA). The PCR protocol included an

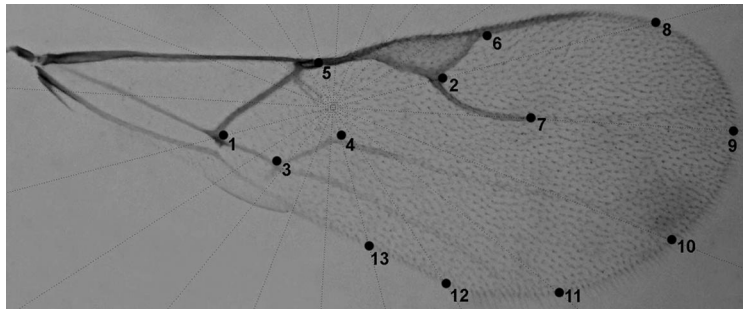


Fig. 1. *Praon abjectum* forewing with marked landmarks and semilandmarks.

initial denaturation at 95°C for 5 min, 35 cycles consisting of 1 min at 95°C, 1 min at 54°C, 2 min at 72°C, and a final extension at 72°C for 10 min.

The Second Expansion Segment of the 28S rRNA gene (28S D2) was amplified using the forward primer 28SD2f (5′-AGAGAGAGTTCAAGAGTACGTG-3′) (Belshaw and Quicke 1997) and the reverse primer 28SD2r (5′-TTGGTCCGTGTTTCAAGACGGG-3′) (Campbell et al. 1993). Each PCR reaction was carried out in a volume of 20 μl, containing 1 μl of extracted DNA, 14.35 μl of H_2O, 2 μl of High Yield Reaction Buffer A (with 1 × Mg), 1.5 μl of $MgCl_2$ (2.25 mM), 0.5 μl of dNTP (0.25 mM), 1 μl of each primer (0.5 μM), and 0.15 μl of KAPA*Taq*DNA polymerase (0.0375U/μl) (Kapa Biosystems Inc.). The PCR protocol included an initial denaturation at 95°C for 3 min, 30 cycles consisting of 30 s at 95°C, 30s at 48°C, 2 min at 72°C, and a final extension at 72°C for 10 min. The PCR products were purified using the QIAquick Purification Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. DNA sequencing was performed using automated equipment (Macrogen Inc., Seoul, South Korea).

Phylogenetic Analyses. Sequences of COI and 28S were manually edited in FinchTV (Geospiza, Inc., Seattle, WA, http://www.geospiza.com) and aligned using the Clustal*W* program integrated in MEGA5 (Tamura et al. 2011). Evolutionary distances using Kimura's two-parameter model were calculated to estimate the divergence of different *P. abjectum* biotypes. Using the Modeltest 3.7 program (Posada and Crandall 1998), the general time reversible (GTR + I + Γ) model of nucleotide substitution was estimated as the best fit model of sequence evolution based on the Akaike Information Criterion (AIC). Phylogenetic relationships were reconstructed using Bayesian inference (BI). BI analysis was conducted with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003), using a Markov chain Monte Carlo iteration for five million generations, trees being sampled every 100 generations. The first 12,500 trees were discarded as burn-in. To confirm the convergence of the parameters we used the program Tracer v1.5.0 (Rambaut and Drummond 2007) and the program FigTree 1.3.1. (Rambaut 2006–2009) to view the consensus tree with posterior probabilities.

Morphometric Analyses. The wing morphology and shape are of adaptive significance and could be related to the varying ecological roles and physiological constrains of the insects flight (e.g., Berwaerts et al. 2002). To explore and quantify the morphological variation, the wing shape of 63 *P. abjectum* females that emerged from three aphid hosts were analyzed by means of geometric morphometrics. Samples consisted of three biotypes: 1) *P. abjectum* that parasitizes *A. sambuci* (44 specimens from Serbia and 3 specimens from Czech Republic), 2) *P. abjectum* that parasitizes *L. trirhodus* (6 specimens from Montenegro), and 3) *P. abjectum* that parasitizes *Rhopalosiphum padi* (10 specimens from Czech Republic). *P. abjectum* specimens are generally scarcely present in the field. For this reason, the samples submitted to morphometric analyses differed somewhat from those used for molecular analyses. For the morphometric analyses we could include additional specimens that were well preserved in dried condition in the coauthors' collections, but unsuitable for DNA extraction.

The right forewing of each specimen was photographed using a LEICA microscope at 50× magnification and a CANON digital camera. To describe the wing shape, we used a combination of seven landmarks and six semilandmarks (positions and definitions are given in Fig. 1). All landmarks and semilandmarks were digitized using the Tps-Dig software (Rohlf 2005) by the same person (A.M.B.). Before digitizing, semilandmarks were defined using MakeFan6 (see Fig. 1) to ensure a consistent placement of semilandmarks at equal angular dis-placements along the curves. Landmarks were superimposed by the generalized Procrustes analysis, which eliminates variation because of the scale, position, and orientation of landmark configurations (Rohlf and Slice 1990, Bookstein 1991). Semilandmarks were superimposed by allowing them to slide along curves bounded by landmarks to minimize the Procrustes distances among individuals (Bookstein 1997). Superimposition of semilandmarks was conducted using SemiLand6. The programs MakeFan6 and Semiland6 belong to the IMP series (Sheets 2003).

Wing size was computed as the centroid size (CS), which is the measure of the size in geometric morphometrics and reflects the amount of dispersion around the centroid of the landmark configuration. To explore the variation in the size of the wings among the three biotypes of *P. abjectum*, we performed an analysis of variance (ANOVA) on the centroid size. To

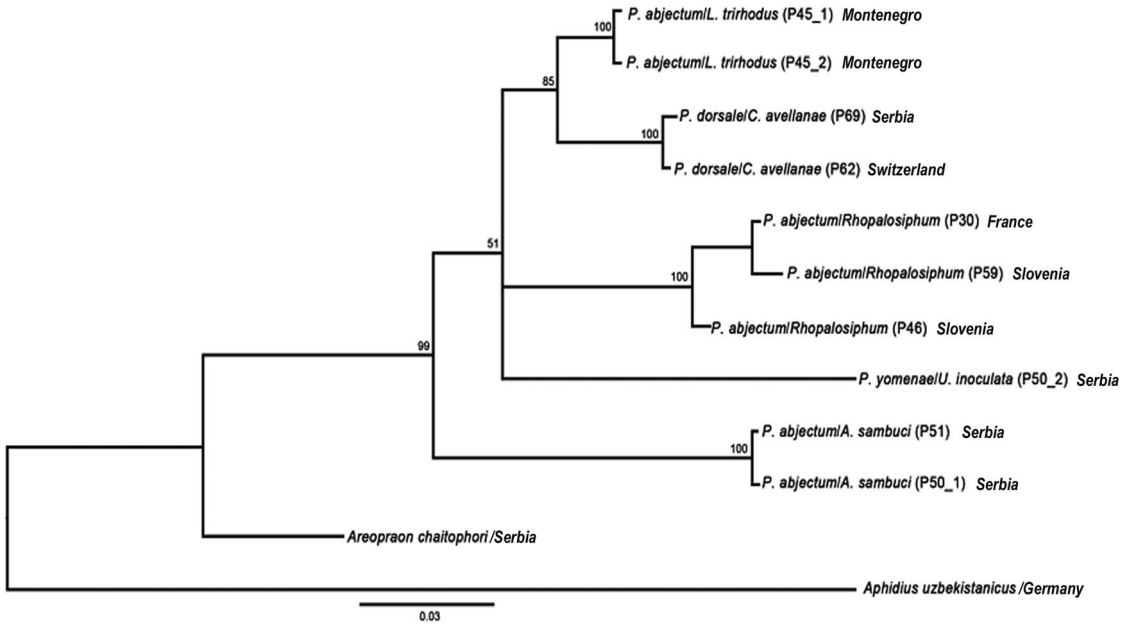


Fig. 2. BI phylogram for COI mtDNA sequences of the species belonging to the genus *Praon* showing Bayesian posterior probabilities above 50%. Scale bar indicates substitutions per site (0.03). COI mtDNA were also amplified for the following outgroups, *Areopraon chaitophori*, a species belonging to the same tribe Praini and *Aphidius uzbekistanicus* belonging to the same subfamily Aphidiinae.

analyze the variation in wing shape of parasitoids from different hosts we performed a MANOVA analysis on the full set of shape variables (Zelditch et al. 2004).

Differences among parasitoid biotypes emerging from different aphid hosts were explored by discriminant function (DF) analysis. We report both original and cross validation percentages of the cases correctly classified by DF analysis. A leave-one-out cross-validation test was applied, which uses the available data for cross validation avoiding the problems of random selections. The comparison of both percentages quantifies the uncertainty in assigning individuals to a certain group according to the estimated DF (Manly 1997). The divergence in the shape of the wings between hosts is visualized by Canonical Variate Analysis (CVA).

Results

Phylogenetic Analysis. In Fig. 2, we present the phylogenetic tree with Bayesian posterior probabilities (PP) for the mitochondrial COI sequences. All species from the genus *Praon* clustered together within the same group with 99% probability. Two specimens of *P. abjectum* originating from the association of *A. sambuci*/*S. nigra* were separated with 100% posterior probability from the other *Praon* taxa that formed a poorly supported phyletic group (PP = 51%). Within this group, aside a *Praon yomenae* as a separate taxon, three well-supported clades (all PP = 100%) were formed by 1) *P. abjectum* in association with the aphid host from the genus *Rhopalosiphum*, 2) *P. abjectum* from *L. trirhodus*, and 3) *P. dorsale* associated with a hazelnut aphid, *Corylobium avellanae* (Fig. 2).

A comparison of the COI mtDNA sequences between *P. abjectum* biotypes using Kimura's two-parameter model showed substantial genetic divergence. Distances between the biotype associated with *L. trirhodus* and those associated with *Rhopalosiphum* sp. and *A. sambuci* were 5.4–6.5 and 7.7%, respectively, while the distance between the latter two biotypes ranged from 9.5 to 10%. These distances are far beyond any intraspecific distances typically observed for COI in Aphidiinae (Derocles et al. 2012).

On the BI tree constructed for the 28SD2 sequences, the congeneric species showed a close relatedness by grouping within the same clade (Fig. 3). Further clustering within the *Praon* clade is evident with two subclades formed, but with low posterior probabilities (PP = 66–68%). Species grouped within the first subclade are *P. abjectum* in association with *Rhopalosiphum* sp., *P. dorsale* from *C. avellanae* and *P. abjectum* from *L. trirhodus*. The second *Praon* subclade clustered *P. yomenae* in association with *Uroleucon inoculata* and *P. abjectum* from *A. sambuci*.

Pairwise comparisons of the nuclear sequences show a closer relatedness between the *P. abjectum* biotypes associated with *Rhopalosiphum* and *L. trirhodus*, with a distance of 0.1%, whereas the *A. sambuci* biotype differed by 0.5 and 0.6% from the previous two, respectively.

Geometric Morphometrics. The ANOVA showed no significant differences in wing size between specimens of the three *P. abjectum* biotypes (model df =

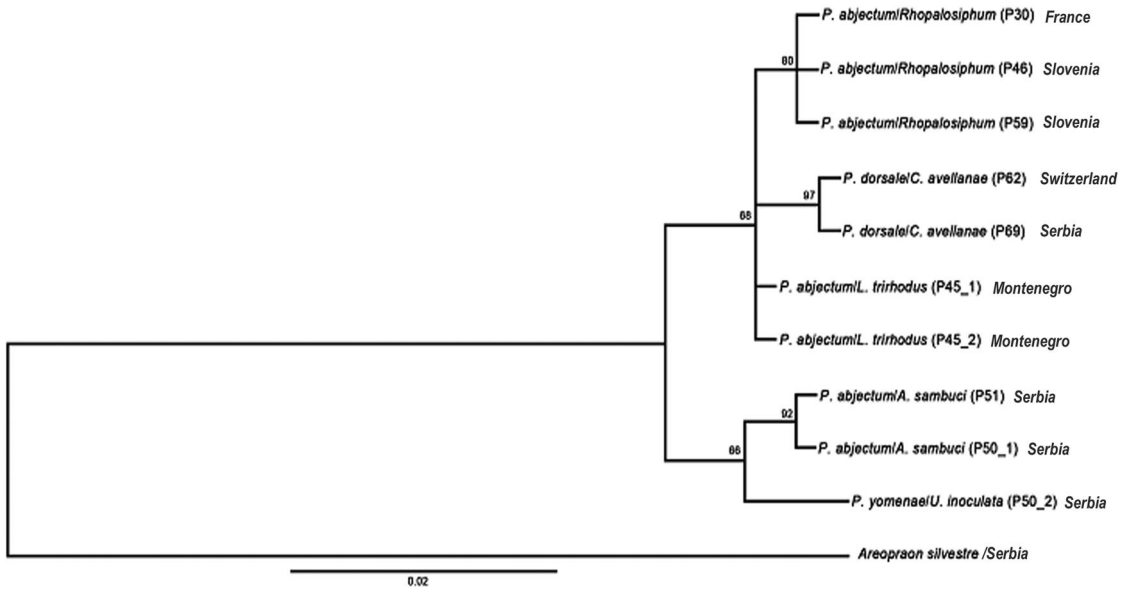


Fig. 3. BI phylogram for 28SD2 sequences of the species belonging to the genus *Praon* showing Bayesian posterior probabilities (values above 50%). Scale bar indicates substitutions per site (0.02). *Areopraon silvestre* belongs to the same tribe Praini and is used as an outgroup to root the tree.

2; error df = 54; $F = 1.07$; $P = 0.3511$). However, there was a highly significant difference in the shape of the wings among biotypes (MANOVA: Wilks' $\lambda = 0.1177$; df1 = 44; df2 = 66; $P < 0.0001$). The divergence in wing shape expressed as Procrustes and Mahalanobis distances is provided in Table 2.

The following percentages were found for the classification of individuals per biotype (the first and the second value in brackets being the original and cross-validation, respectively): *A. sambuci* 97% (63.4%), *L. trirhodus* 100% (33.33%), and *R. padi* 90% (20%).

To explore the divergence in wing shape among the three biotypes, we performed a canonical variate analysis (CVA), which reduces the within group variances and increases the between group divergences in the shape of the wing. *P. abjectum* specimens emerged from *L. trirhodum* were clearly discriminated from the ones associated with *A. sambuci* and *R. padi* by the shape of the stigma (described by landmarks 2, 5, and 6) and the position of the radial vein (described by landmarks 2 and 7) (Fig. 4). In addition, the specimens of the *Longicaudus* biotype have a wider distal part of the wing, especially the area above the radial nerve (described by landmarks 2, 6, 7, and 8), and narrower proximal part of the wing (described by landmarks 1, 3, 4, and 5). *P. abjectum* specimens emerged from *A. sambuci* and *R padi* were discriminated along the second CV axis. The main shape changes that discriminated these two groups are related to the stigma shape and the position of the radial nerve. The biotype from *R. padi* has an elongated and narrower stigma (described by landmarks 2, 5, and 6) and an elongated radial nerve orientated laterally (described by landmarks 2 and 7) compared with the *A. sambuci* biotype with a more triangular stigma and shorter radial nerve orientated toward the posterior margin of the wing.

Table 2. Procrustes distances (below diagonal) and Mahalanobis distances (above diagonal) between the mean wing shapes of three biotypes

Host species	*Aphis sambuci*	*Longicaudus trirhodus*	*Rhopalosiphum padi*
Aphis sambuci		**5.5903**	**2.7180**
Longicaudus trirhodus	**0.0274**		**5.8527**
Rhopalosiphum padi	**0.0165**	0.0214	

The statistical significance of differences in mean shape of specimens that emerged from different hosts was estimated by permutation test based on 10.000 iterations. The statistically significant differences ($P < 0.05$) are shown in boldface type.

Description of Two New *Praon* Species

Praon longicaudus Tomanović & Starý n. sp.
(Figs. 5–11)

Diagnosis. On the basis of the number of antennal segments (16 and exceptionally 17) and wing venation patterns (colored m-cu vein and colorless Rs + M vein), the new species is close to *P. abjectum.* However, *P. longicaudus* n. sp. differs from *P. abjectum* in having a slightly concave dorsal margin of ovipositor sheaths (straight dorsal margin in *P. abjectum*), two conical spines at the tip of ovipositor sheaths (tip of ovipositor sheaths in *P. abjectum* without conical spines), and a more elongated petiole (T1 liters/w = 1.60–1.65 times in *P. longicaudus* n. sp. versus T1 liters/w = 1.30–1.40 in *P. abjectum*).

Female. *Head.* Malar space equal to 0.23–0.25 of longitudinal eye diameter. Clypeus oval with 18–20

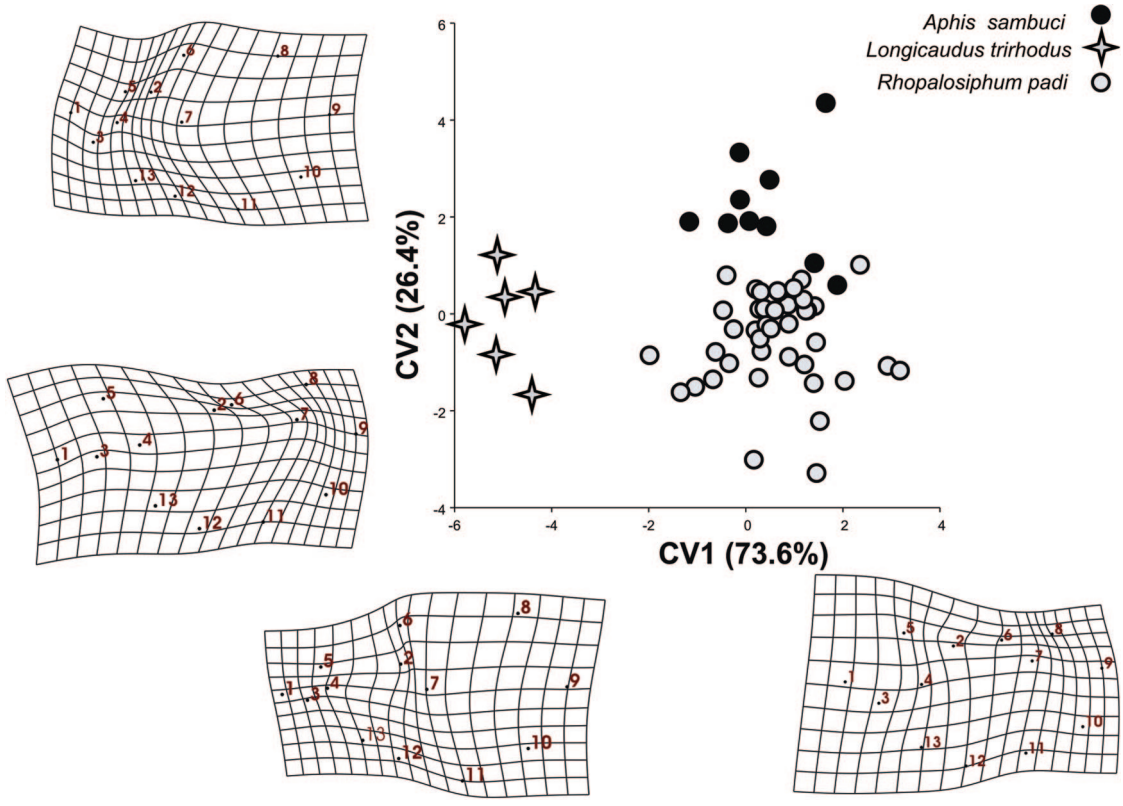


Fig. 4. The position of the specimens in the morphospace defined by first and second CV axis. The shape changes along CVA are presented as the thin-plate spline deformation grids. The shape changes are exaggerated by a factor 3. (Online figure in color.)

long setae. Tentorial index 0.22–0.30. Maxillary palps 4-segmented, labial palps 3-segmented (Fig. 5). Head 1.25–1.40 times wider than mesoscutum. Antennae 16-(17)-segmented, filiform, with semierect setae which are shorter than the diameter of the segments (Fig. 6). Flagellomere 1 (F1) and 2 (F2) elongate, 4.20–5.00 times as long as wide, and 2.60–3.00 times as long as wide, respectively (Fig. 6). F1, 1.25–1.40 times longer than F2. F1without, and F2 with 0–1 longitudinal placodes.

Mesosoma. Mesonotum (Fig. 7) with central lobe densely covered with long setae. Lateral lobes of mesonotum with small hairless areas. Notaulices deep and distinct throughout. Propodeum (Fig. 8) smooth, densely pubescent except for small upper and lower central parts that are hairless.

Forewing. Stigma 3.45–3.75 times as long as wide (Fig. 9) and 1.50–1.80 times as long as distal abscissa of R_1 (=metacarpus). m-cu vein colored and Rs + M vein colorless throughout. Forewing M vein long and colorless.

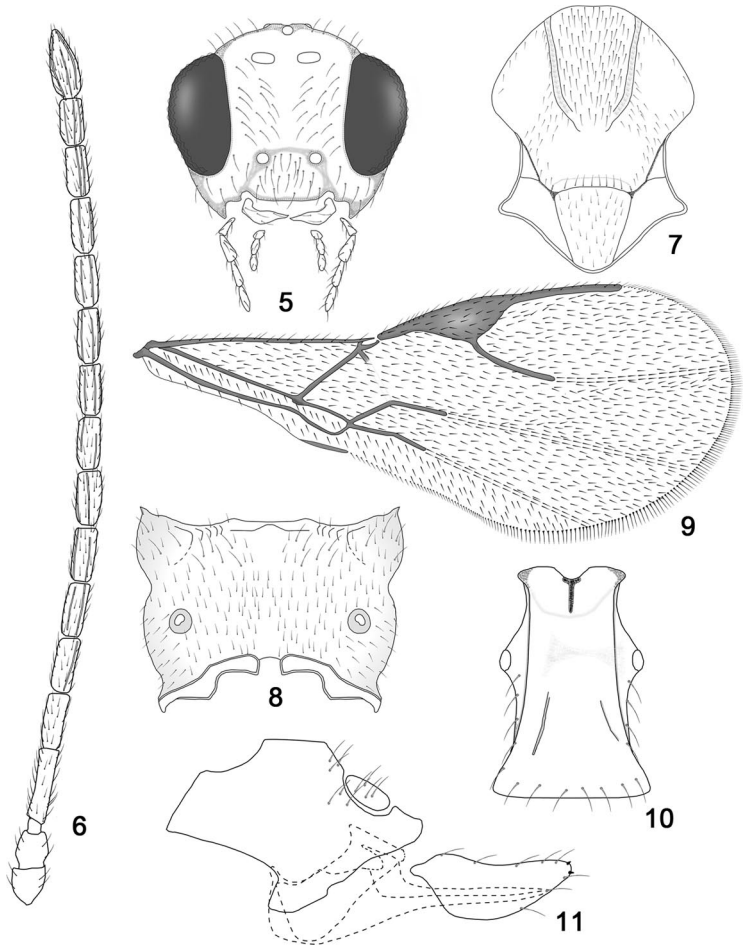
Metasoma. Petiole elongate 1.60–1.65 times as long as wide (Fig. 10), at the level of the spiracles. The distance between spiracles and apex less than the width at the spiracles level. Petiole with long setae along the sides. Ovipositor sheath (Fig. 11) elongate with dorsal margin slightly concave. Ovipositor sheath with round apex, with two conical spines on its upper and lower edge (Fig. 11).

Coloration. Head black. Scape and pedicel yellow to light brown. Only narrow ring at the base of F1 yellow, remaining part of antennae brown. Mouthparts yellow to brownish. Petiole light brown to yellow. Legs yellow with dark apices. Metasoma brown. Remaining body parts black.

Body Length. 1.8–2.0 mm.

Male. Antennae 18-segmented. Petiole shorter than in female, 1.30–1.50 times as long as wide, at the level of the spiracles. Mouthparts and legs yellow to brown. Petiole brown. Remaining body parts black.

Type Material. HOLOTYPE: 1 ♀, Montenegro, Mt. Durmitor-Inđina dolina, 14–VII–2000, reared from *L. trirhodus* on *T. aquilegifolium*, leg. Ž. Tomanović. Deposited in coll. of Belgrade Natural History Museum, Serbia. Acc. No. KC128672. PARATYPES: 2 ♀ 2 ♂, slide mounted, Montenegro, Mt. Durmitor–Pištaline, 01–VII–2001, reared from *L. trirhodus* on *T. aquilegifolium*, leg. Ž. Tomanović; 1 ♀, slide mounted, Montenegro, Mt. Durmitor-Inđina dolina, 14–VII–2000, reared from *L. trirhodus* on *T. aquilegifolium*, leg. Ž. Tomanović; 1 ♀, slide mounted, Montenegro, Mt. Durmitor–Skakala, 02–VII–2002, reared from *L. trirhodus* on *T. aquilegifolium*, leg. Ž. Tomanović; 1 ♀, slide mounted, Montenegro, Mt. Durmitor, 04–VIII–2011,



Figs. 5–11. *P. longicaudus* sp. n. (♀). (5) Head (frontal view). (6) Antenna. (7) Mesonotum (dorsal view). (8) Propodeum (dorsal view). (9) Forewing. (10) Petiole (dorsal view). (11) Ovipositor sheath (lateral view).

reared from *L. trirhodus* on *T. aquilegifolium*, leg. Ž. Tomanović; 1 ♀, in ethanol, Montenegro, Plav-Oko, 21–VII–2006, reared from *L. trirhodus* on *T. flavum*, leg. Ž. Tomanović. All paratypes are deposited in coll. of Faculty of Biology, Institute of Zoology, Belgrade.

Expected Distribution. *P. longicaudus* n. sp. is probably following the distribution of the specific association of *L. trirhodus*/*T. aquilegifolium*, thus could be expected in the Palaearctic and possibly Nearctic. To date, *P. longicaudus* was collected from submontane and montane areas of Montenegro.

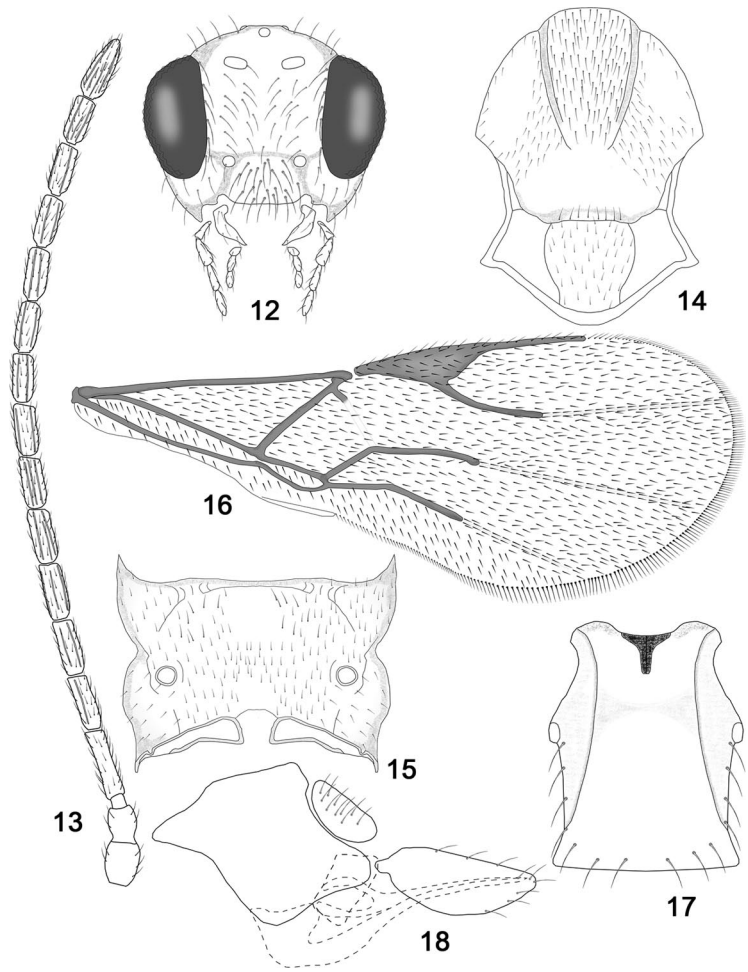
Etymology. The new species takes its name after the host aphid *L. trirhodus.*

Praon sambuci Tomanović & Starý n. sp.
(Figs. 12–18)

Diagnosis. Morphologically, *P. sambuci* n. sp. is very close to *P. abjectum* and both species share the following common characters: wing venation pattern, absence of conical spines at the tip of ovipositor sheath and straight dorsal margin of ovipositor sheath. In addition, both species are parasitizing aphids from the genus *Aphis.* The new species *P. sambuci* n. sp. differs by having a more triangular stigma than *P. abjectum* (proportion between length and width stigma in *P. sambuci* n. sp. is 3.00–3.50 vs. 3.50–4.00 in *P. abjectum*) and a smaller number longitudinal placodes on flagellomere 2 (1–2 in *P. sambuci* n. sp. vs. 2–3 in *P. abjectum*).

Female. *Head.* Malar space equal to 0.20–0.30 of longitudinal eye diameter. Clypeus oval with 18–20 long setae. Tentorial index 0.30–0.36. Maxillary palps 4-segmented, labial palps 3-segmented (Fig. 12). Head ≈1.3 times wider than mesoscutum. Antennae 16-segmented, filiform, with semierect setae that are shorter than the diameter of the segments (Fig. 13). F1 and F2 elongate, 4.00–4.50 times as long as wide, and 2.50–2.90 times as long as wide, respectively (Fig. 13). F1, 1.30–1.40 times longer than F2. F1without, and F2 with 1–2 longitudinal placodes.

Mesosoma. Mesonotum (Fig. 14) with central lobe densely covered with long setae. Lateral lobes of mesonotum with large hairless areas. Notaulices deep and distinct throughout. Propodeum (Fig. 15) smooth,



Figs. 12–18. *P. sambuci* sp. n. (♀). (12) Head (frontal view). (13) Antenna. (14) Mesonotum (dorsal view). (15) Propodeum (dorsal view). (16) Forewing. (17) Petiole (dorsal view). (18) Ovipositor sheath (lateral view).

densely pubescent except for small upper and lower central parts that are hairless.

Forewing. Stigma 3.50 times as long as wide (Fig. 16) and 1.40–1.50 times as long as distal abscissa of R_1 (=metacarpus). m-cu vein colored and Rs + M vein colorless throughout (Fig. 16). Forewing M vein long, pigmented in the first part and with spectral remaining parts, which reaching almost forewing margin.

Metasoma. Petiole elongate 1.25–1.35 times as long as wide (Fig. 17), at the level of the spiracles. The distance between spiracles and apex less than the width at the spiracles level, with long sparse setae along the petiole sides. Ovipositor sheath (Fig. 18) with a straight dorsal margin. Round apex, without conical spines on its upper and lower edge.

Coloration. Head black. Scape and pedicel yellow. Only narrow ring at the base of F1 yellow, remaining part of antennae brown. Mouthparts yellow. Petiole light brown to yellow. Legs yellow with dark apices. Metasoma brown. Remaining body parts black.

Body Length. 1.7–2.4 mm.

Male. Antennae 18-segmented, filiform. Mouthparts and legs yellow to brown. Annelus and narrow ring at the base of F1 yellow. Petiole yellow to brown. Remaining body parts black.

Type Material. HOLOTYPE: 1 ♀, Serbia, Kragujevac–Vraćevšnica, 16–VI–2011, reared from *A. sambuci* on *S. nigra*, leg. A. Mitrovski–Bogdanović. Deposited in coll. of Belgrade Natural History Museum, Serbia. Acc. No. KC128674. PARATYPES: 36 ♀ 5 ♂, Serbia, Kragujevac–Vraćevšnica, 16–VI–2011, reared from *A. sambuci* on *S. nigra*, leg. A. Mitrovski–Bogdanović. All paratypes are deposited in coll. of Faculty of Biology, Institute of Zoology, Belgrade.

Other material: 3 ♂, Serbia, Kovilovo, 16–V–1993, reared from *A. sambuci* on *S. nigra*, leg. Ž. Tomanović; 1 ♂, Serbia, Belgrade–Topčider, 26–V–2000, reared from *A. sambuci* on *S. nigra*, leg. Ž. Tomanović; 3 ♀, Czech Republic, 1984, reared from *A. sambuci* on *S. nigra*, leg. P. Starý.

Expected Distribution. Currently, *P. sambuci* n. sp. was collected throughout the Palaearctic from the

aphid host *A. sambuci* in association with *S. nigra* and *S. ebulus.* Aside the plausible Palaearctic distribution, presence of the newly described species could be expected in North America as well. Though the host range is still to be investigated, most probably the *P. sambuci* n. sp. will follow the distribution of *A. sambuci*/ *Sambucus* sp. associations.

Etymology. The new species takes its name after the host aphid *A. sambuci.*

Discussion

One of the proposed mechanisms of cryptic speciation within the subfamily Aphidiinae is the splitting of sympatric populations driven by host specialization and changes in host selection behavior of the parasitoid (Tremblay and Pennacchio 1988, Rehman and Powell 2010). A good example of such a scenario represents *Aphidius ervi* (Braconidae: Aphidiinae), a common biocontrol agent of the pea aphid across the Palaearctic. Populations of this parasitoid associated with the stinging nettle aphid, *Microlophium carnosum,* were determined to express strong host specialization and to have evolved reproductive isolation from *A. ervi,* where after this parasitoid was described as a new cryptic species, *Aphidius microlophii* (Pennacchio and Tremblay 1987).

Several new species within the *Praon dorsale*/*yomenae* group have been described recently from the Eastern Mediterranean, which represent good examples of cryptic speciation by specialization to different, plant host-associated aphids of the genus *Uroleucon* (Mescheloff and Rosen 1988; Tomanović et al. 2003a,b).

Based on the results of molecular analyses and morphometrics, we identified two new cryptic species here: *P. abjectum*-like parasitoids in association with *A. sambuci*/*S. nigra* and *L. trirhodus*/*T. aquilegifolium* are described (according to their aphid host) as *P. sambuci* n. sp. and *P. longicaudus* n. sp., respectively.

According to Kavallieratos et al. (2005) and Tomanović et al. (2006), *P. abjectum* is recognized as a sister taxon to the remaining *Praon* species, possessing several plesiomorphic character states (e.g., absence of conical apical spines at the top of ovipositor sheaths and straight dorsal margin of the ovipositor sheaths). The newly described species *P. sambuci* shares the same plesiomorphic characters, whereas *P. longicaudus* possesses two conical apical spines at the top of the ovipositor sheaths and a slightly concave dorsal margin of the ovipositor sheaths. Based on the wing shape *P. longicaudus* n. sp. is clearly distinguished from *P. sambuci* n. sp. and *P. abjectum.* The main shape changes that discriminate all three biotypes are related to the stigma shape and the position of radial nerve.

The nuclear gene 28SD2 is a more conservative genetic marker than mitochondrial DNA, thus had mostly been used in phylogenetic analyses of higher taxa within the family Braconidae (Quicke 2002, Dowton et al. 2002, Whitfield et al. 2002). The low genetic variation of 28SD2 sequences determined in this study between species of the genus *Praon* was to be expected and only confirmed their close relatedness. However, using the mitochondrial COI, clearly identified three taxa within the *P. abjectum* group as separate entities with substantial genetic divergence among them, that is, *P. abjectum* in association with *Rhopalosiphum* sp., *P. abjectum* associated with *A. sambuci*/*S. nigra* and *P. abjectum* in association with *L. trirhodus*/*T. aquilegifolium.*

P. longicaudus n. sp. is morphologically and genetically clearly separated from *P. abjectum.* However, the large genetic distance between *P. sambuci* n. sp. and *P. abjectum* (9.5–10% at COI) is not matched by morphological variation, including the shape of the forewings. Both species are morphologically very close, with slight differences in the shape of the stigma and the number of placodes on flagellomere 2. Because the relatively small number of *P. sambuci* n. sp. specimens in our analysis could have affected the estimation of the mean shape and variance in geometric morphometrics (Cardini and Elton 2007), a larger sample size would certainly increase the reliability and therefore the correct morphological identification.

A. sambuci and *L. trirhodus* are holocyclic and heteroecious aphids of Palaearctic distribution (Blackman and Eastop 2006), both being reported as invasive pests in the United States (Foottit et al. 2006). Pike et al. (2000) documented that *A. sambuci* in the northwestern United States is parasitized by *Lysiphlebus testaceipes,* a native North American parasitoid species. However, parasitoid guilds of *A. sambuci* in Europe are comprised of *Binodoxys angelicae, B. acalephae, Ephedrus plagiator, P. abjectum,* and *Lysiphlebus cardui* (Kavallieratos et al. 2004b, Starý 2006). Our examination of the *P. abjectum* specimens collected from Southeastern and Central Europe associated with *A. sambuci* showed that in fact they belong to a separate taxon, the here described *P. sambuci.* For this reason, all literature records of *P. abjectum* on common elder aphid hosts should be re-examined for the possible presence of the newly described cryptic species. Parasitoids associated with *L. trirhodus* in North America are still unknown, whereas in Europe the following four species were recorded to parazitize this aphid (Kavallieratos et al. 2004b): *Aphidius eglanteriae, Ephedrus laevicolis, P. abjectum,* and *Trioxys chaetosiphonis.* Analyzes of *P. abjectum* specimens collected from *L. trirhodus*/*T. aquilegifolium* in Southeastern Europe identified them as a separate species, *P. longicaudus.* In the absence of any native parasitoids effectively controlling populations of the invasive pests *A. sambuci* and *L. trirhodus* in North America, it could be considered to include parasitoids from Europe in classical biological control programs. As a reported member of the parasitoid guilds for both aphid hosts, one of the potential agents might be *P. abjectum.* For this reason, it is of great importance to pursue the host range tests to define the level of host specialization of the newly described species within the *P. abjectum* group and to ascertain accordingly what distribution patterns are to be expected.

There is a great interest in resolving the taxonomic status of aphid parasitoids to estimate the role of each separate species in the very complex interactions in agroecosystems and the surrounding natural habitats (Kavallieratos et al. 2008, Tomanović et al. 2009). Our study shows that an integrative approach using molecular markers, classical morphology, and geometric morphometrics shows great potential to resolve cryptic taxa of aphid parasitoids in agreoecosystems as well as natural environments.

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