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Agriotypus armatus Curtis, 1832, a parasitoid of *Silo pallipes* Fabricius, 1781: the first record for the Balkan Peninsula

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ABSTRACT

Key-words: *Agriotypus, parasitoid, Serbia, distribution, ecological parameters*

Among the diverse order of Hymenoptera, aquatic species are of special interest. During macrozoobenthos sampling in the rivers of Serbia in 2003 and during the period 2011–2012, pupae of *Silo pallipes* Fabricius, 1781 were recorded which were parasitized by an ichneumonid wasp *Agriotypus armatus* Curtis, 1832, this being the first record of it for the Balkan Peninsula. A total of 217 *A. armatus* specimens were collected at 29 localities situated along 16 watercourses of Serbia. All four parasitoid stages (egg, larva, pupa and resting adult) were recorded. The identity of the parasitoid was confirmed using standard molecular methods. This study also focuses on the ecology of the parasitoid and its host. The widespread distribution of *A. armatus* in Europe suggests the presence of this species in other Balkan countries, but this has not yet been confirmed due to a lack of surveys and/or the constantly increasing pollution of freshwaters.

RÉSUMÉ

Agriotypus armatus Curtis 1832, un parasitoïde de *Silo pallipes* Fabricius, 1781 : premier enregistrement dans la péninsule des Balkans

Mots-clés : *Agriotypus, parasitoïde, Serbie, distribution, paramètres écologiques*

Parmi l'ordre diversifié des hyménoptères, les espèces aquatiques sont d'un intérêt particulier. Pendant un échantillonnage du macrozoobenthos dans les rivières de Serbie en 2003 et au cours de la période 2011–2012, des nymphes de *Silo pallipes* Fabricius, 1781 ont été remarquées parasitées par une guêpe ichneumonide, *Agriotypus armatus* Curtis 1832, ce qui est le premier enregistrement de cette espèce pour la péninsule balkanique. Un total de 217 échantillons d'*A. armatus* ont été collectés dans 29 localités situées le long de 16 cours d'eau de Serbie. Les quatre stades du parasitoïde (œuf, larve, nymphe et adulte) ont été observés. L'identité du parasitoïde a été confirmée en utilisant des méthodes moléculaires classiques. L'étude met également l'accent sur l'écologie du parasitoïde et de son hôte. La large diffusion de *A. armatus* en Europe suggère la présence de cette espèce dans d'autres pays des Balkans, mais cela n'a pas encore été confirmé en raison d'un manque d'études ou de la pollution sans cesse croissante des eaux douces.

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The order of Hymenoptera represents one of the largest groups among insects, with over 60 000 parasitic species (Grissell, 1999). This very diverse group also includes some aquatic representatives (150 species from 11 families, Bennett, 2008) parasitizing insect larvae or other arthropods living in water. Their hosts are mainly aquatic insects from the following orders: Odonata, Coleoptera, Hemiptera, Diptera and Trichoptera.

The subfamily Agriotypinae (family Ichneumonidae) is monotypic with *Agriotypus* as the only genus, containing 16 described species (Bennett, 2001) in the Palearctic and Oriental biogeographic regions. All species are ectoparasitic idiobionts of Trichoptera prepupae and pupae from the families Goeridae, Odontoceridae and Uenoidae (Konishi, 1994). *Agriotypus armatus* Curtis, 1832 is the only registered European species.

The most common host of *A. armatus* is *Silo pallipes* Fabricius, 1781 (Trichoptera, Goeridae), a typical species of rocky streams and small rivers, with widespread distribution in Europe. Its life cycle lasts one year; adults appear mainly in June and July, and sporadically in May or August (Elliott, 1982). Females lay their egg clumps below the water surface, attaching them to larger stones. Larvae of *S. pallipes* use small pebbles to construct their cases, in which they also pupate. Pupation takes place in spring and lasts a relatively short time, probably less than one month (Elliott, 1982). The life cycles of the parasitoid and its host are well synchronized. Female wasps oviposit their eggs in the host case at times when the host is in an inactive stage, *i.e.*, in the prepupal and pupal stage. Parasitoid larvae undergo five instars; the first instar is morphologically clearly different from the rest, having a long bifurcate caudal process at the end of the abdomen. After consuming all of the internal body contents of its host, the last larval instar of the parasitoid spins its cocoon within the host case including a spectacular respiratory filament. Parasitoids mature in fall, overwintering in the Trichoptera cases as resting adults until the emergence period.

The aim of our research was to investigate the distribution of *Agriotypus armatus* on the territory of Serbia. In addition, some ecological information on *A. armatus* is given.

Sampling of *Silo pallipes* larvae and pupae occurred in 2011 (April, July, September, October and December) and 2012 (February, May) in rivers of Serbia situated within two zoogeographic regions (*sensu* Illies, 1978): regions V (the rivers Rača, Rasina, Raška and Studenica) and VII (the rivers Crnica, Mlava, Radovanska Reka and Vrla). In addition, *A. armatus* was registered in 2003 (February, April, May, July, August and October) and in 2012 (August) in eight rivers from three zoogeographic regions: regions V (the rivers Banjska Reka, Djerekarska Reka and Toplica), VI (the rivers Pusta Reka and Veternica) and VII (the rivers Božica, Jerma and Ribarska Reka). The material was collected using a Surber net (sampling area of 300 cm^2), and by picking up *Silo pallipes* larvae and pupae with tweezers. Samples were preserved in 96% alcohol. All sampling sites (296 to 1056 m a.s.l.) were situated within epirhithral and metarhithral stream zones with mean water temperatures of 12.24 ± 0.42 °C. The mean concentration of oxygen was 10.06 ± 0.16 $mg \cdot L^{-1}$, pH was 7.86 ± 0.06 and conductivity was 0.38 ± 0.01 $mS \cdot cm^{-1}$. Sediments consisted of bedrock, boulders and gravel. Mean concentrations of total phosphorus, orthophosphates and ammonium were 0.04 ± 0.005, 0.02 ± 0.002 and 0.17 ± 0.01 $mg \cdot L^{-1}$, respectively. Physical and chemical parameters were measured directly in the field using a water field kit PCE-PHD meter (Germany) and in the laboratory of the Institute of Chemistry, Technology and Metallurgy, Belgrade, Serbia.

Identification of specimens was conducted using the keys of Waringer and Graf (1997), Bennett (2001) and Lechthaler and Stockinger (2005) in the laboratory of the Institute of Zoology, Faculty of Biology, University of Belgrade. All cases containing *S. pallipes* pupae (with or without a respiratory filament) were opened in order to obtain accurate numbers of eggs, larvae, pupae and resting adults of *A. armatus*.

Six specimens of *Agriotypus armatus* were used for molecular confirmation of its species status and to get preliminary insight in to its genetic variability (Table I). DNA was extracted from each individual specimen (stored in 96% ethanol) using a KAPA Express Extract kit (Kapa Biosystems) following the manufacturer's instructions. The barcoding region of the mitochondrial cytochrome oxidase subunit I (COI) gene was amplified using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). DNA amplification and purification were performed as

Table I
Specimens used for molecular identification.

Species	River	Locality	Date	Accession number
Agriotypus armatus	Vrla	Vlasina	12 September, 2011	KJ748543
Agriotypus armatus	Raška	Novi Pazar	30 November, 2011	KJ748540
Agriotypus armatus	Studenica	Golija	30 August, 2011	KJ748542
Agriotypus armatus	Radovanska Reka	Boljevac	07 October, 2011	KJ748541
Agriotypus armatus	Rasina	Mitrovo polje	02 March, 2012	KJ748539
Agriotypus armatus	Mlava	Žagubica	11 July, 2011	KJ748538

described in Petrović *et al.* (2013), while DNA sequencing was performed by Macrogen Inc. (Seoul, Korea). Sequences were edited using FinchTV (www.geospiza.com) and aligned by CLUSTAL W integrated in the MEGA5 software (Tamura *et al.,* 2011) Sequences showed no indels (insertion/deletion). Sequences of *A. armatus* were trimmed to a length of 551 bp. All sequences are deposited under the accession numbers KJ748538-KJ748543 in GenBank. For calculation of average genetic distances between sequences of *A. armatus* we used Kimura's two-parameter method (K2P) of base substitution. Neighbor-joining (NJ) and maximum-likelihood (ML) trees were obtained using the MEGA5 software. The robustness of the trees was assessed using bootstrap analysis with 1000 replicates. A COI sequence of *Orthocentrus* sp. from GenBank (Accession No. FJ414847.1) was used as an outgroup taxon for molecular phylogenetic analyses.

Parasitized pupae were found at 29 localities in 16 watercourses belonging to zoogeographic regions V, VI and VII (Figure 1; Appendix).

Of the total number of 1187 *Silo pallipes* cases examined, 650 cases (54.76%) were empty, 331 (27.89%) were non-parasitized and 206 (17.35%) were parasitized. In the Crnica river, 99 parasitoids were sampled at five localities along the river course, which is in keeping with the great number of hosts there (Table II). This number represents 48.06% of the total number of parasitoids collected in all rivers studied from April 2011 to May 2012. On the other hand, in the Radovanska Reka and Rasina rivers only a small number of parasitoids were found, which was in accordance with the small number of hosts collected there (three (1.46%) and one (0.48%) specimen, respectively).

Only the rivers studied during 2011 and 2012 (February and May) were included for calculating the percentage of parasitized cases because the rivers studied in 2003 (the rivers Banjska Reka, Djerekarska Reka, Jerma, Pusta Reka, Ribarska Reka, Toplica and Veternica) and in August of 2012 (the Božica) were sampled only sporadically. Empty cases were excluded from the analysis. The percentage of parasitized trichopteran larvae is shown in Figure 2. The highest percentage of parasitism was noted in the Raška river (50 specimens; 52.63%), and the lowest one in the Rasina river (one specimen; 7.69%). Although a large number of Trichoptera cases were collected in the Studenica river, only a small percentage of them (11 specimens; 14.1%) were parasitized.

The total numbers of developmental stages of *Agriotypus armatus* collected in Serbian rivers are presented in Figure 3. In only two rivers, Crnica and Raška, were all stages of the parasitoid found (Figure 4). The samples collected in spring (May) in the Mlava, Crnica and Raška rivers contained parasitoid eggs (Figure 4a). The highest number of individuals of *A. armatus* was found in the larval (78 specimens; 37.86% of the total number of parasitoids) and resting adult (72 specimens; 34.95%) stages. First larval instars were identified on the basis of their long caudal appendages (Figure 4b), which are missing in later instars (Figures 4c, 4d). Parasitoid pupae (Figure 4e) were collected mostly from August to October (occasionally also in May in the Crnica river), but from October to February only resting adults (Figure 4f) were found in trichopteran cases.

Partial sequences of the COI gene obtained from six Serbian specimens of *A. armatus* were subjected to BLAST analysis and compared with reference specimens obtained from the National Center of Biotechnology Information (http://www.ncbi.nim.nih.gov). BLAST analysis

Figure 1
Distribution of Agriotypus armatus on the territory of Serbia (white circles) 1-4, Rača; 5-8, Studenica; 9-11, Raška; 12, Rasina; 13, Djerekarska Reka; 14, Toplica; 15, Banjska Reka; 16, Ribarska Reka; 17-21, Crnica; 22-24, Mlava; 25, Radovanska Reka; 26, Pusta Reka; 27, Veternica; 28, Jerma; 29, Vrla; 30, Božica.

Table II
Total numbers of collected Silo pallipes cases (empty, non-parasitized or parasitized).

Rivers/Total number	Crnica	Mlava	Rača	Radovanska Reka	Rasina	Raška	Studenica	Vrla
***S. pallipes* cases**	467	179	108	14	38	185	168	28
Empty cases	243	127	53	8	25	90	90	14
Non-parasitized host	125	31	39	3	12	45	67	9
Parasitized host	99	21	16	3	1	50	11	5

showed that there were no significant similarities between the submitted sequences and the reference specimens. The similarity of *A. armatus* with some ichneumonid genera (*Microcharops*, *Gelis*, *Stenomacrus, Orthocentrus* and subfamily Pimplinae) was up to 85%. In GenBank there exists only one unverified sequence of the barcoding region for the *Agriotypus* sp. COI gene (Accession number JF962477). This sequence shows more than 99% identity with our sequences. Calculated K2P distances (0–0.9%) show that there were no significant differences between *A. armatus* specimens from different rivers. The topology of both ML and NJ trees showed that all specimens of *A. armatus* form one phylogenetic clade (Figure 5).

In addition to the European species of *Agriotypus armatus*, there are also 15 species of the genus *Agriotypus* known from the Oriental region probably due to the abundance of Goeridae species which number up to 110 (out of 140) in this region (Armitage and Arefina-Armitage, 2011). Compared with the European species, the Oriental parasitoid species display a very

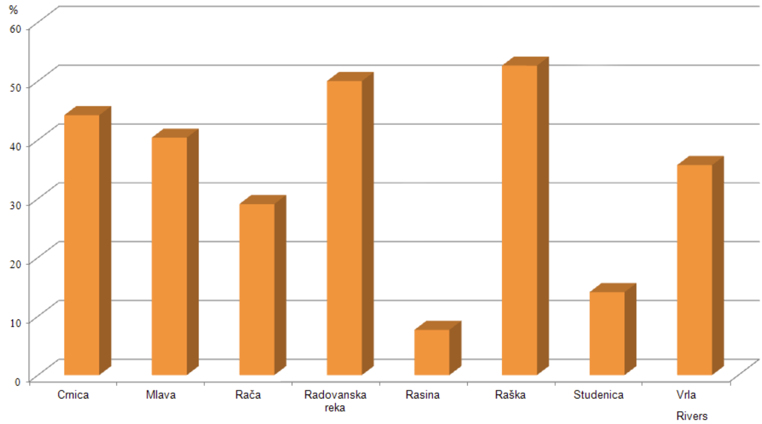

Figure 2
Percentage of parasitized Silo pallipes pupae parasitized by Agriotypus armatus in Serbian rivers.

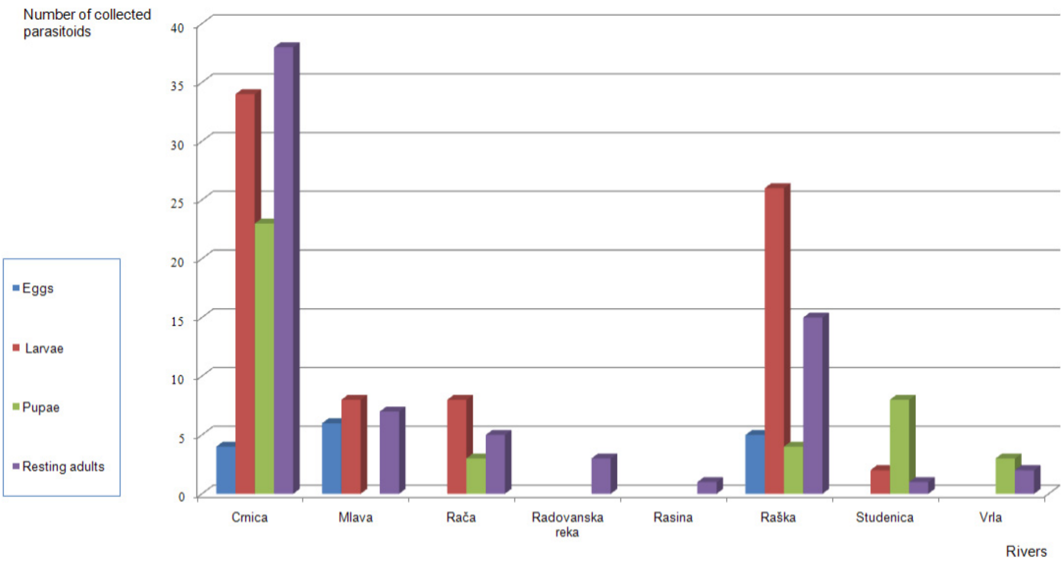

Figure 3
Numbers of developmental stages (eggs, larvae, pupae, adults) of A. armatus collected in Serbian rivers.

narrow range of specific host types which restricts their geographical area (Bennett, 2001). The widespread distribution of *A. armatus* on the other hand, reflects its broad spectrum of available hosts consisting of 12 different species (Yu *et al.*, 2012).

In previous studies dealing with macrozoobenthos of Serbian rivers (Radovanović, 1931, 1953; Baračkov, 1973; Marinković–Gospodnetić, 1975; Simić, 1993; Paunović, 2001; Živić, 2005; Savić *et al.*, 2013), only *Silo pallipes* adults and larvae were reported. In the present study, *Agriotypus armatus*, a widespread species in Europe, was registered in Serbia for the first time, with stable parasitoid populations, especially in the Crnica and Raška rivers. Almost equal numbers of larvae and resting adults were collected, which implies that a large number of individuals finish their development.

In the Crnica river (sampling stations CR4 and CR5) *Silo pallipes* larvae were significantly clumped, mainly on larger stones (>20 cm). This clumping provides for easier host finding. According to Elliott (Elliott, 1983) an increase in clumped *Silo* pupae raises the mean density of pupae parasitized by *A. armatus*. It follows that host grouping probably caused the high parasitoid records at these localities.

The finding of parasitoids in the pupal stage at CR4 and CR5 in May is unusual because at this time of the year only resting adults are expected. A possible explanation is that some

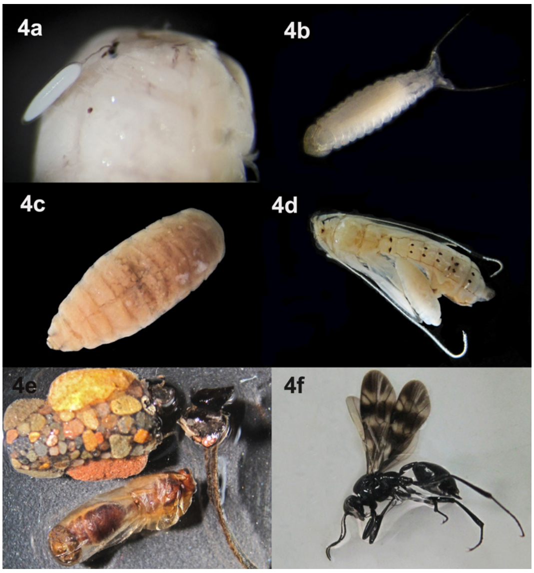

Figure 4
Developmental stages of Agriotypus armatus. 4(a) Parasitoid egg on host prepupa; 4(b) parasitoid larva (first instar); 4(c) parasitoid larva (later instar); 4(d) parasitoid larva on host pupa; 4(e) parasitoid pupa and host case; 4(f) parasitoid adult.

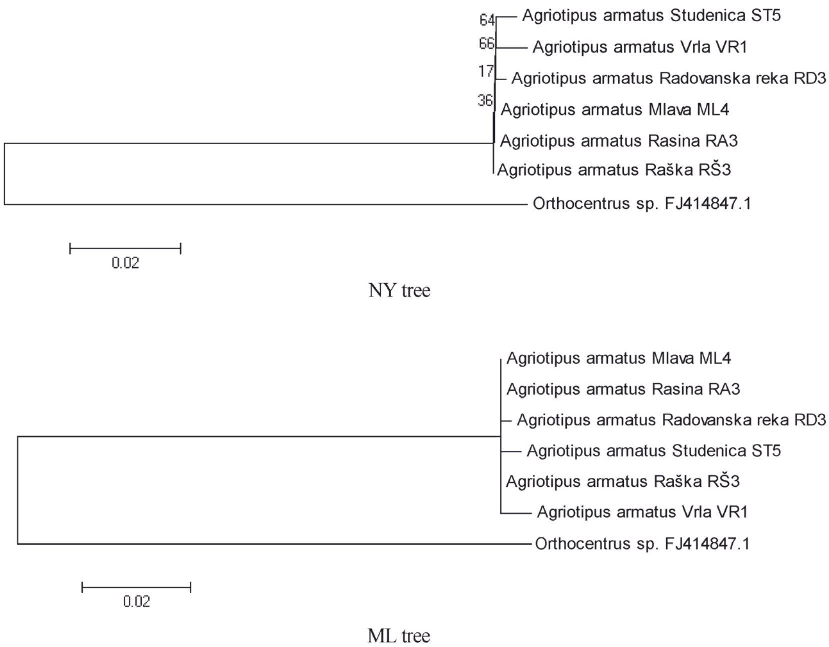

Figure 5
Optimal neighbor-joining (NY) tree and maximum-likelihood (ML) tree with the highest log likelihood (-1046.4106) of Serbian specimens of Agriotypus armatus.

specimens in the population can overwinter in the pupal stage; or, more likely, development was stopped in the previous year due to hyperparasitism or unfavorable ecological conditions. Chemical parameters generally were in their normal range, except for nutrient concentrations. Values of dissolved oxygen were not below 7 $mg \cdot L^{-1}$, but somewhat lower values were observed in the summer period when the water temperature was higher. Parasitized specimens of *Silo pallipes* were mostly found in waters with temperatures between 12 and 15 °C, but occasionally also in colder environments (locality RA4: 4.9 °C, locality CR1: 9.7 °C) or warmer ones; for example, in the Studenica river in the summer period (locality ST6: 18 °C, locality ST3: 19.8 °C). The concentrations of total phosphorus and orthophosphates indicate somewhat higher values than those characteristic of clean waters (from 0.005 to 0.02 mg·L^{-1}; Dulić, 2010). This was also true of ammonium concentrations. Considerably higher ammonia and phosphate concentrations were recorded at localities situated downstream of trout farms, where organic compounds increased water pollution. Contrary to these findings, the concentrations at localities upstream of trout farms were below the level of detection.

Inasmuch as the biology of the host and that of the parasitoid are well known, it is unsurprising that more detailed studies on parasitizing rates and stability of *Silo pallipes* populations have not been conducted since the survey of Elliott (Elliott, 1982, 1983). Host-parasite interactions in a broader context should be emphasized to understand their importance in water ecosystems better (Kohler, 2008). It is known that environmental conditions such as host food availability, habitat structure, temperature and other ecological factors (Sheridan *et al*., 2000) affect host-parasitoid relations. Some future studies should focus on this relationship between the availability of resources to the host and the rate of parasitism.

It is also important to expand our knowledge on distribution patterns of this species in neighboring Balkan countries. Of interest here are the localities on the rivers Božica and Jerma (Figure 1) which are situated near the border with Bulgaria; and that on the Rača river, which is situated near the border with Bosnia and Herzegovina; these examples suggest the possibility that the parasitoid is also present in neighboring Balkan countries where *Silo pallipes* and other representatives of the family Goeridae are registered. Its presence can be observed even at more northerly locations within zoogeographic region XI. The small number of individuals registered in certain rivers might be due to either insufficent investigation or increasing organic pollution in mountain streams and rivers.

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APPENDIX

Collecting sites of parasitized *Silo pallipes* pupae in Serbia. Abbreviations of collectors: IŽ = Ivana Živić; DS = Dalibor Stojanović; KB = Katarina Bjelanović, ZM = Zoran Marković.
Banjska Reka river (BK1); Božica river (BŽ1); Crnica river (CR1, CR3, CR4, CR5, CR6); Djerekarska Reka river (DJ2); Jerma river (JE7); Mlava river (ML3, ML4, ML5); Pusta Reka river (PR11); Rača river (RČ1, RČ4, RČ5, RČ6); Radovanska Reka river (RD3); Rasina river (RA4), Raška river (RŠ1, RŠ2, RŠ3); Ribarska Reka river (RI1); Studenica river (ST1, ST3, ST5, ST6); Toplica river (TI1); Veternica river (VE4); Vrla river (VR1).

> *ZOOGEOGRAPHIC REGION V – DINARIC WESTERN BALKAN:*

BK1, 28 April 2003, 2 pupae., leg. IŽ; DJ2, 25 October 2003, 1 pupa., leg. IŽ; RČ1, 16 October 2011, 2 pupae., leg. KB; RČ4, 10 December 2011, 1 pupa., leg. KB; RČ4, 6 May 2012, 2 pupae., leg.KB; RČ5, 20 June 2011, 1 pupa., leg. KB; RČ5, 27 September 2011, 3 pupae., leg. DS; RČ5, 10 December 2011, 1 pupa., leg. KB; RČ5, 6 May 2012, 1 pupa., leg. KB; RČ6, 27 September 2011, 2 pupae, leg. DS; RČ6, 16 October 2011, 2 pupae, leg. KB; RČ6, 6 May 2012, 1 pupa., leg. KB; RA4, 2 March 2012. 1 pupa., leg. DS; RŠ1, 15 May 2012, 5 pupae, leg. KB; RŠ2, 25 June 2011, 24 pupae., leg. KB; RŠ2, 10 October 2011, 1 pupa., leg. KB; RŠ2, 30 November 2011, 5 pupae., leg. DS; RŠ2, 15 May 2012, 1 pupa., leg. KB; RŠ3, 25 June 2011, 7 pupae., leg. KB; RŠ3, 29 August 2011, 1 pupa, leg. KB; RŠ3, 30 November 2011, 2 pupae., leg. DS; ST1, 30 August 2011, 2 pupae, leg. DS; ST3, 30 August 2011, 1 pupa, leg. DS; ST5, 30 August 2011, 4 pupae, leg. DS; ST6, 30 August 2011, 1 pupa, leg. DS; TI1, 7 August 2003, 1 pupa, leg IŽ.

> ZOOGEOGRAPHIC REGION VI – HELLENIC WESTERN BALKAN:

PR11, 3 February 2002, 1 pupa, leg. IŽ; VE4, 1 May 2003, 1 pupa, leg. IŽ.

> ZOOGEOGRAPHIC REGION VII – EASTERN BALKAN:

BŽ1, 9 August 2012, 5 pupae, leg. ZM; CR1, 19 April 2011, 1 pupa, leg. DS; CR1, 13 July 2011, 3 pupae, leg. DS; CR1, 5 September 2011, 3 pupae, leg. KB; CR1, 4 December 2011, 4 pupae, leg. KB; CR1, 12 May 2012, 6 pupae, leg. KB; CR3, 13 July 2011, 2 pupae, leg. DS; CR3, 6 October 2011, 1 pupa, leg. KB; CR3, 12 May 2012, 5 pupae, leg. KB; CR4, 19 April 2011, 5 pupae, leg. DS; CR4, 5 September 2011, 1 pupa, leg. KB; CR4, 6 October 2011, 18 pupae, leg. KB; CR4, 4 December 2011, 3 pupae, leg. KB; CR4, 23 February 2012, 1 pupa, leg KB; CR4, 12 May 2012, 12 pupae, leg. KB; CR5, 13 July 2011, 3 pupae, leg. DS; CR5, 5 September 2011, 8 pupae, leg. KB; CR5, 6 October 2011, 11 pupae, leg. KB; CR5, 12 May 2012, 10 pupae, leg. KB; CR6, 12 May 2012. 2 pupae, leg. KB; JE7, 1 August 2003, 1 pupa, leg. IŽ; ML3, 9 May 2012, 1 pupa, leg. KB; ML4, 11 July 2011, 1 pupa, leg. DS; ML4, 15 October 2011, 3 pupae, leg. KB; ML5, 11 July 2011, 1 pupa, leg. DS; ML5, 15 October 2011, 1 pupa, leg. KB; ML5, 9 May 2012, 14 pupae, leg. KB; RD3, 7 October 2011, 3 pupae, leg. KB;. RI1, 31 July 2003, 1 pupa, leg. IŽ; VR1, 12 September 2011, 4 pupae, leg. DS; VR1, 4 October 2011, 1 pupa, leg. KB.