Natural history, immature stage morphology, and taxonomic status of the threatened skipper *Pyrgus cinarae* (Rambur, 1839) in the Iberian Peninsula (Lepidoptera: Hesperiidae)

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**Abstract.** New data on the ecology and natural history of the rare skipper *Pyrgus cinarae* (Rambur, 1839) in the Iberian Peninsula are presented and the habitats of its populations in Central Spain are described. *Filipendula vulgaris* Moench (Rosaceae) is the larval foodplant in the Serranía de Cuenca (Iberian Mountain System), while in the Sierra de Ávila (Central Mountain System) the larva feeds on *F. vulgaris* and *Potentilla recta* L. (Rosaceae). We report *Dolichogenidea sicarius* (Marshall, 1885) (Hymenoptera: Braconidae: Microgastrinae) as a larval parasitoid. Details of the egg, the last instar larva, and the pupa are shown in scanning electron micrographs. The egg has particularly high radial ribs, the last instar larva has setae with crowned or pointed tips and barrel-like cuticular formations, and the pupa has hairy mesothoracic tubercles. Differences with respect to immature stages of *Pyrgus cacaliae* (Rambur, 1839) are discussed; the types of larval head capsule setae and the shape of pupal mesothoracic tubercles are of special taxonomic value. In the light of morphological and genetic data we conclude that the recently discovered population from Sierra de Ávila can be ascribed to subspecies *clorinda* (Warren, 1927), due to genetic and morphological similarity to the Serranía de Cuenca populations. The main threats to its populations are overgrazing or the abandonment of agricultural practices. Finally, we discuss the conservation status of the species in the study area.

**Introduction**

The distribution of *Pyrgus cinarae* (Rambur, 1839) reaches into Central Asia but in Europe is limited to the Iberian and Balkan Peninsulas showing a clearly disjunct pattern (Hernández-Roldán et al. 2011a). In the Iberian Peninsula it is found in small and
isolated populations and is considered one of the rarest butterflies (Hernández-Roldán & Vicente 2010). The extremely localized Iberian populations are found in Central Spain, more precisely in the Iberian Mountain System (Serranía de Cuenca and Sierra de Albarracín) (Chapman 1901; Querci 1932; Gómez-Bustillo & Fernández-Rubio 1974; Fernández-Rubio 1981; Redondo 1990; Arce et al. 2006; Hernández-Roldán & Vicente 2010), where subspecies clorinda (Warren, 1927) was described, and in the Central Mountain System (Sierra de Ávila) where it has recently been discovered (Hernández-Roldán & Vicente 2010; Vicente & Parra 2010). The latter population is separated by ca. 300 km from the nearest populations in the Iberian Mountain System.

Given the geographical position, it is also doubtful whether the new populations could be assigned to subspecies clorinda or they represent a new subspecies. The Iberian populations are separated by ca. 1800 km from their nearest conspecifics living in the Balkan Peninsula (Greece, Macedonia, and Bulgaria). The species is also recorded from Ukraine, Turkey, Armenia, and from southern Russia to Turkistan (Tolman & Lewington 1997; Gorbunov 2001; Kudrna 2002; Gorbunov & Kosterin 2003). The Iberian P. cinarae have been found to be sister populations to those existing outside of Iberia, from which they are genetically well diverged (2.6%, Hernández-Roldán et al. 2011a). They appear to have been isolated for around one million years, being considered by Hernández-Roldán et al. (2011a) an independent evolutionary lineage.

The species’ life history has been recently described and immature stages figured by Wagner (2009), using material from Greece. The larva feeds on Potentilla recta (Wagner 2009) or P. hirta (Wagner pers. com.) and females lay their eggs on the fruits. Winter is spent as a first instar larva inside the egg and emergence takes place during the following March. The larva feeds on the leaves until full size is reached and pupation takes place at the end of May, with the pupal stage lasting for around three weeks. A detailed morphological study of the eggs, larvae and pupae has not been previously undertaken for this species.

At European level, the conservation status of Pyrgus cinarae has been considered as least concern (LC) following IUCN criteria (Van Sway et al. 2010). In Spain, as there are relatively few populations, the species has been considered vulnerable (Hernández-Roldán et al. 2011c) using criteria B1ac(iii) (IUCN 2001). The populations from Castilla-La Mancha Region (Serranía de Cuenca) have been listed under the category “interés especial” (special interest, DOCM 2006). Moreover, most of the populations in the Iberian Mountain System live within the Serranía de Cuenca Natural Park and are therefore legally protected.

The objective of the present paper is to describe the life cycle and the morphological details of the egg, last instar larva and pupa of Pyrgus cinarae in the Iberian Peninsula. The morphological study is based on scanning electron micrographs. We also discuss the taxonomic status of the new populations found in the Central Mountain System and evaluate the threats and conservation status of the species in the study area.

Abbreviations
MNCN: Natural History Museum, Madrid, Spain
Material and Methods

Sample collecting and rearing experiments

Three populations were studied in Central Spain during the years 2010 and 2011, two of them located in Serranía de Cuenca (Huélamo and Carrascosa, Iberian Mountain System) and one in Sierra de Ávila (Central Mountain System). Eggs were collected by following females in the field until oviposition took place and then searching for them on the plant. Larvae were collected on the foodplants. Rearing was done from field-collected eggs and larvae, under laboratory conditions, with the weather parameters different from the natural habitat (higher temperature and lower altitude and relative humidity). Larvae were fed using the same species of plants on which they were collected. The adults used in this study were reared from eggs or larvae or were studied in the collections of the authors or in the Museo Nacional de Ciencias Naturales (Natural History Museum, Madrid, Spain – MNCN).

Scanning electron micrographs

Images used to study the morphology of the egg, last instar larvae, and pupae were obtained using a Hitachi S-3000N scanning electron microscope, with an acceleration voltage of 20 kV. Dried samples were used in the case of the egg and pupa. The eggs were killed with ethyl acetate when the larva was already formed inside the egg to prevent the collapsing of the egg when dried. For the study of pupae, exuviae were used after the adult emergence. Larvae were preserved in 70% ethanol and then fixed with 1.5% formaldehyde. For larval dehydration a series of increasing concentrations of ethanol was used up to absolute ethanol, ending with critical-point drying using Emitech K850. Samples were then coated with a 10 nm layer of gold using an Au-Cr Quorum 150TS sputter. The microscope and the rest of the used equipment are located in the Servicio Interdepartamental de Investigación (SIDI) of the Universidad Autónoma de Madrid. A scale bar was added to all obtained images. Egg measurements were performed directly on the images using ImageJ1.43 software (Ferreira & Rasband 2010).

Results

Studied Material

Pyrgus cinarae clorinda (Warren, 1927)

Pyrgus cinarae (Rambur, 1839)


Dolichogenidea sicarius (Marshall, 1885) (Hymenoptera: Braconidae: Microgastrinae)


Habitats and natural history in the Iberian Peninsula

Habitats of P. cinarae in the Iberian Peninsula are located at altitudes ranging from 1100 to 1500 m and consist of xeromesophilous grasslands with extensive livestock management, in the supramediterranean bioclimatic stage (Figs 1, 2). The populations in the Serranía de Cuenca (Iberian Mountain System) belong to the Central Iberian Mediterranean botanical biogeographical region and the population from the Sierra de Ávila to the Occidental Iberian Mediterranean region (Rivas Martínez 1987). The populations in the Serranía de Cuenca are found in the calcicole Portuguese oak (Quejigo) forest vegetation communities: Huélamo in the phytosociological association Violo willkommii-Quercetum fagineae (Braun-Blanquet & Bolós 1950) and Carrascosa in the association Cephalanthero longifoliae-Quercetum fagineae (Rivas Goday 1959). The population in the Sierra de Ávila lives in silicicole Pyrenean oak (Melojo) forests, in the association Luzulo forsteri-Quercetum pyrenaicae (Rivas Martinez 1962).
The foodplant in the Serranía de Cuenca is *Filipendula vulgaris* Moench (Rosaceae) (Figs 3, 4) in the two studied localities and in the Sierra de Ávila the species uses *F. vulgaris* and *Potentilla recta* L. (Rosaceae) (Fig. 5). Females lay their eggs one by one on the dried fruits of *P. recta* (heads of achenes) or *F. vulgaris* (polyachenes) (Figs 6, 7). After four or five days the larvae are already developed but remain inside the eggshell, where they overwinter. In the following spring they hatch during the months of March and April (Fig. 8). Larvae feed on the leaves of their foodplants (Fig. 9) and build shelters to protect themselves against predators and parasitoids (Figs 10, 11). These shelters are built by joining leaves of the plant with silk threads produced by the larval silk glands. The third instar larvae are attacked in the Sierra de Ávila by the solitary endoparasitoid *Dolichogenidea sicarius* (Marshall, 1885) (Hymenoptera: Braconidae: Microgastrinae) (Figs 12, 13). At the end of June or in July larvae reach their maximum development and pupate inside a shelter made with leaves of the plant or near it (Figs 14, 15). The pupal phase (Figs 16, 17) lasts for around three weeks, after which the adults emerge during July and August (Figs 18–21).

**Immature stage morphology**

*Egg* (Figs 6, 7, 22–29)

The egg is whitish after being laid and subsequently turns to a pale orange colour in one or two days. Its form is spherical with hollows in the base and annular areas. The diameter is $0.86 \pm 0.03 \, \text{mm} \, (n=5)$ and its height $0.64 \pm 0.02 \, \text{mm} \, (n=4)$. The chorion surface is perforated by pores (Figs 24, 25) and the egg is covered by radial and transversal ribs that form cells, usually of rectangular shape (Figs 24, 25). When the radial ribs meet in the annular area the cells have a pentagonal form (Fig. 23). Aeropyles (respiratory openings of the egg) are found in the junctions of radial and transverse ribs (Figs 24, 25). In the equator of the egg the number of radial ribs is $22–24 \, (n=5, \, \text{Fig. 26})$. The radial ribs are much higher ($32 \pm 2 \, \mu\text{m}, \, n=5$) than the transverse ribs (Fig. 24). In the annular area of the egg, the micropylar rosette has seven to nine ($n=5$) polygonal petaloid cells (Fig. 28). Surrounding the micropylar rosette there are three to four series of cells, so that the total number of cells in this area is $49$ to $53 \, (n=5, \, \text{Fig. 27})$. The micropylar depression is pentagonal and three to five ($n=5$) micropylar openings are found in the angles (Fig. 29), although the most usual number is five.

*Last instar larva* (Figs 14, 15, 30–37)

The surface of the head capsule displays a reticular pattern and is covered by a large number of thin setae of different lengths, with smooth surface and a toothed crown on their tip (Fig. 31). The collar is placed after the head capsule (Fig. 30) and has a smooth cuticle and setae of different lengths. In the rest of the body segments the cuticle is armoured by star shaped formations (Fig. 34). The first thoracic segment has one trichobotrium on each side (Fig. 33). Each segment of the thorax, the abdomen and the collar has a couple of special barrel-like cuticular formations (Fig. 35). These formations were first described by Hernández-Roldán et al. (2011b) in *Pyrgus*.
cacaliæ (Rambur, 1839). In addition, the dorsal and subdorsal areas of each segment bear thin and short setae with a crowned tip and a lower number of long and thin setae with pointed tips. The setae of the latter type are more abundant in the subdorsal area (Fig. 33). Both crowned and pointed setae have a rounded base. Spiracles are placed between the subdorsal and lateral zones on the third thoracic and on the abdominal segments (Fig. 36). The inner part of the spiracles has prominent papillae with spiny branches. The anal plate (Fig. 37), which is placed on the last abdominal segment, is smoother than the rest of the cuticle and has a large number of crown-tipped setae of different lengths.

Pupa (Figs 16, 17, 38–45)
The pupa is bullet shaped, with a smooth cuticle of dark grey or black colour. It is externally covered by wax giving the pupa a characteristic pruinose appearance and a light grey colour (Figs 16, 17). It has mesothoracic tubercles on each side of the anterior border of the mesothorax (Fig. 38). The external wall of the tubercles is undulated (Fig. 39) and the tubercle itself has a dense cover of hairs (Figs 39, 40). The pretubercular chamber has short hairs in the area that is close to the tubercle and spines in the area that is far from it (Fig. 40). The pupal cuticle has scattered long setae with a rounded base that are denser on the dorsal and lateral areas of the abdomen (Figs 38, 43). On each segment of the subdorsal zone of the abdomen there are spiracles (Fig. 41) that are filled with spiny-branched papillae (Fig. 42). The last abdominal segment bears the cremaster (Figs 43, 44), which is formed by around 30 setae with coiled helicoid tips. These setae keep the pupae fixed to the substrate by means of the silken threads spun by the larvae (Fig. 45).

Adult external morphology
Males from the recently discovered population in the Sierra de Ávila (Figs 46–49) have a shiny yellow ochre colour and veins clearly visible and lined in pale yellow on the hindwing underside. The males from the Serranía de Cuenca display the same pattern (Figs 50–53). Females from the Sierra de Ávila (Figs 54–56) are similar, as far as these characters are concerned, to those from the Serranía de Cuenca (Figs 57–59). Nevertheless, females show a greater variability than males in both populations. Subspecies clorinda could be separated from oriental samples belonging to the nominal subspecies (Figs 60, 61) mainly because in the latter the hindwing underside shows a “very dull ochre” colour (Warren 1927).

Discussion
Habitat and natural history
The life cycle of Pyrgus cinarae is similar in the Iberian Peninsula and Greece (Wagner 2009). It shows specific and infrequent ecological adaptations: it is the only European
Pyrgus whose females lay eggs on the fruits and one of only three species, together with P. carlineae (Rambur, 1839) and P. cirsii (Rambur, 1839), that overwinter as a larva inside the eggshell. The similar life history traits of Iberian and oriental populations support the hypothesis that, although they have been isolated for around a million years (Hernández-Roldán et al. 2011a), no evidence for the establishment of potential barriers to gene flow (other than geographic) exist that could indicate speciation according to the biological species concept.

In Greece, the habitat of the species consists of grasslands that are extensively grazed by sheep and goats. Here the species could be possibly threatened by overgrazing or by the abandonment of extensive grazing (Wagner 2009). The Iberian populations are extremely localized and seem to occupy a narrower ecological niche than the oriental populations (Hernández-Roldán et al. 2011a). In the Serranía de Cuenca the grasslands with Filipendula vulgaris that could be favourable for the species are rare (López 1978). This fact, together with the false idea that the habitat for the species consisted of “extremely arid areas, devoid of vegetation” and that the larval foodplants were “herbaceous Poaceae” (Gómez-Bustillo & Fernández-Rubio 1974; Viedma & Gómez-Bustillo 1985), could have been the cause of the few records of the species. This contrasts with the fact that, at the beginning of the 20th century, Orazio Querci and his wife were able to collect almost a hundred individuals in the area (Warren 1927; Querci 1932). It is therefore possible that new sampling performed on the basis of our recent knowledge of life history traits, together with the information provided by ecological niche modelling based on environmental variables (Romo et al. 2006; Hernández-Roldán et al. 2011a), could lead to the discovery of new populations that will improve our knowledge of the species distribution.

**Taxonomic value of immature stage morphology**

When comparing the morphological characters of the immature stages of P. cinarae (see results) with those of P. cacaliae described by Hernández-Roldán et al. (2011b), we have detected some differences. The eggs of P. cinarae are larger than those of P. cacaliae (0.86 mm vs. 0.79 mm) and the radial ribs taller (32 μm vs. 4 μm). Moreover, the cells in the micropylar area have lower numbers in this species (49–53) when compared with P. cacaliae (59–62). Larvae of P. cinarae have crown-tipped setae on the head capsule and branched spiracular papillae, while in P. cacaliae the setae on the head capsule have pointed tips and the spiracular papillae are not branched. Pupal differences consist of the undulated external wall and the hairy surface of the mesothoracic tubercles, pretubercular chamber with hairs and spines and spiracles with branched papillae in P. cinarae; in P. cacaliae the mesothoracic tubercles have smooth walls and lack hairs, the pretubercular chamber has only spines and the spiracles have unbranched papillae. It is therefore evident that the immature stages of P. cinarae have different characters to those of P. cacaliae described in Hernández-Roldán et al. (2011b). This fact supports the use of traits of the egg, larva and pupa for taxonomical purposes and opens the possibility of using them as tools to infer phylogenetic relationships in the genus Pyrgus.
Figs 30 – 37. Scanning electron micrographs of the last instar larva of *Pyrgus cinaracae clorinda* (Sierra de Ávila, Spain). 30. Head capsule and collar. 31. Detail of the head capsule setae and sculpture. 32. Cuticular formations on thoracic and abdominal segments. 33. Detail with different types of subdorsal and lateral setae on the first thoracic segment: large pointed-tip setae, short setae with crowned tips and a trichobotrium. 34. Cuticular formations on an abdominal segment. 35. Detail of a barrel-like formation and the cuticular sculpture. 36. Last abdominal spiracle. 37. Last abdominal segments with the anal plate and setae with pointed and crowned tips, both with rounded bases.
Figs 38–45. Scanning electron micrographs of the pupa of *Pyrgus cincarae clorinda* (Sierra de Ávila, Spain). 38. Head and mesothorax with mesothoracic tubercles and long setae. 39. Lateral view of the mesothoracic tubercle. 40. Frontal view of the mesothoracic tubercle. 41. Abdominal spiracle. 42. Detail of the papillae inside the spiracle. 43. Ventral view of the last abdominal segments. 44. Detail of the cremaster and 45. the tips of cremaster setae.
Taxonomic status of the new population in Ávila

The subspecies *clorinda* was described based on specimens collected in the Serranía de Cuenca. Warren (1927) pointed out differences in the external morphology of the adult: “The characteristics which distinguish this race from the type (eastern form) are: the bright yellow ground colour of the underside of the hindwings (invariably a very dull ochre in eastern European, or Asiatic specimens) and the clearly visible nervures of the same, outlined in a light yellow and in consequence contrasting sharply with the ground colour. The bright shade of the ground colour also suffuses the costa and apex of the forewings underside. On the upperside the light marking of the hindwings are slightly buff, or cream in the male, and of a deep, almost ochre shade in the female.” As has been stated above, specimens from Ávila and Cuenca are indistinguishable regarding the adult external morphology. This is also true as far as the morphology of the male genitalia is concerned (Fernández-Rubio 1981; Hernández-Roldán & Vicente 2010). Moreover, the specimens from the Sierra de Ávila and Serranía de Cuenca have identical mitochondrial DNA haplotypes (cytochrome c oxidase subunit I, COI). This contrasts with the high divergence (2.6%) found between the Iberian and oriental populations detected for the same gene (Hernández-Roldán et al. 2011a). In conclusion, since the populations in Ávila and Cuenca are genetically and morphologically similar, the recently discovered population in Sierra de Ávila should be considered as belonging to subspecies *clorinda* (Warren, 1927).

Conservation

Detected threats to the populations of *P. cinarae* in the Iberian Peninsula include habitat destruction, habitat quality loss, agriculture, and overgrazing, as well as afforestation (Hernández-Roldán & Vicente 2010; Hernández-Roldán et al. 2011c). In the Cuenca area, there is a threat of overgrazing by horses in the population of Huélamo, and this is probably the reason why this population has very low numbers of individuals. Grazing by sheep takes place in the habitat of the Carrascosa population. The habitat in the Sierra de Ávila is managed by extensive grazing and is used as hay meadow area. These practices could pose a serious threat for the future survival of the species if management is intensified. Regarding intrinsic threatening factors, the Iberian populations of *P. cinarae* have been historically on the brink of extinction due to a genetic bottleneck that caused a drastic decline in population numbers, followed by a later expansion. This has resulted in an extremely low genetic variability (Hernández-Roldán et al. 2011a) that, coupled with the remarkably restricted range and a very narrow ecological niche of its populations, suggests that this subspecies is prone to extinction. Even if *clorinda* does not represent a different species, the unique genetic pool that it represents makes protection efforts for this taxon advisable.

The species has been considered under the category “vulnerable” in the Spanish Red List of Invertebrates (Hernández-Roldán et al. 2011c), based on geographical criteria (B criteria, IUCN 2001). In this case it was not possible to rely on population abundance or trends to analyse the species’ status (criteria A, C, D or E, IUCN 2001),
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because these data were not available in the study area. We therefore recommend carrying out population studies like those performed for other rare Iberian skippers (Hernández-Roldán et al. 2009). Such studies would provide a more accurate evaluation of the status of this species and allow sound recommendations for conservation management.

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Figs 46 – 61. a. Dorsal view. Pyrgus cinarae clorinda (Figs 46 – 59) and Pyrgus cinarae cinarae (Figs 60, 61). 46 – 49. Males from Siurra de Ávila, Spain, 1350 m, 31.vii.2010 (46 and 47), ex larva 4.vi.2011 (48 and 49). 50. Male from Carrascosa, Serranía de Cuenca, Spain, 1300 m, 24.vii.2010. 51 – 52. Males from Huéjamo, Serranía de Cuenca, Spain, 1200 m, 28.vii.1926. 53. Male from Serranía de Cuenca, Spain, 1200 m, 22.vii.1933. 54 – 56. Females from Siurra de Ávila, Spain, 1350 m, 2.viii.2010, 10.viii.2010, ex ovo 26.i1.2011. 57. Female from Valdecabras, Serranía de Cuenca, Spain, 1200 m, 25.vii.1926. 58. Female from Huéjamo, Serranía de Cuenca, Spain, 1237 m, 29.vii.2007. 59. Female from Carrascosa, Serranía de Cuenca, Spain, 1300 m, 28.vii.2009. 60. Male from Aiotdzorsky range, Armenia, 1800 m, 26.vii.2007. 61. Female from Voghdjaberd village, Armenia, 1500 m, 23.vii.2009. Specimens in Figs 51 and 57 are syntypes. All specimens are shown at real scale.


