

## ***Incurvaria pirinella* sp. nov., a new species of the *vetulella* species-group (Lepidoptera, Incurvariidae) from Bulgaria, with release of DNA barcodes for European species of *Incurvaria***

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**Abstract.** *Incurvaria pirinella* Junnilainen, Kaitila & Mutanen, **sp. nov.** is described from Bulgaria based on specimens collected by netting and artificial light from several low-elevation localities in Bulgaria. The species is morphologically and genetically most similar to *I. triglavensis* Hauder, 1912. Differences between these two species are present in external appearance and genitalia of both sexes. Additionally, *I. pirinella* shows a distance of 4.74% to its nearest neighbour *I. triglavensis* in the standard DNA barcoding marker (COI-5P). We provide preliminary observations of phylogenetic affinities of European *Incurvaria* and briefly discuss habitat preferences of some species. All species have distinct barcodes with minimum K2P divergences between species averaging 7.05% (range 1.2–12.8%). A world checklist of *Incurvaria* Haworth, 1828 is provided and DNA barcodes for all European species are here released. Finally, we document morphological variation in male genitalia within *I. triglavensis* Hauder, 1912.

### **Introduction**

During several entomological expeditions to the southern Pirin mountain range in Bulgaria, the authors JJ and JPK collected specimens of *Incurvaria* Haworth, 1828, which originally were believed to belong to *Incurvaria triglavensis* Hauder, 1912, described from the Triglav mountain in Slovenia. Later, however, we noticed constant differences in the external habitus between our specimens and those of *I. triglavensis* as illustrated by Huemer (1993). We also observed that the Bulgarian taxon occurs in low-elevation habitats with mainly Mediterranean vegetation, whereas other species of the *vetulella*-group prefer alpine habitats in Central and Southern Europe. Therefore, a tissue sample of the Bulgarian taxon was sequenced for the DNA barcode region of the mitochondrial COI gene, which turned out to differ clearly from all other European species of *Incurvaria*. Examination of the genitalia of Bulgarian specimens showed differences from *I. triglavensis* in both male and female genitalia. Therefore, we describe the taxon here as a new species. We also provide DNA barcodes for all European species of *Incurvaria*, as well as for three other European Incurvariidae.

## Material and methods

**Collecting.** The specimens of *I. pirinella* sp. nov. were captured with insect nets during daytime, in the evening and early in the morning, as well as using artificial light late at night. Specimens were stored alive in glass vials and killed in a freezer, after which they were spread.

**Morphological examination.** Genitalia preparations were conducted following standard techniques (Robinson 1976). Although the sclerotised parts are well visible without staining, male genitalia were moderately stained with mercurochrome, because unstained parts become nearly transparent after a few years in Euparal. To make membranous parts of the female genitalia more visible, they were stained with chlorazol-black. The unrolling technique (Nielsen 1980) was applied for some male specimens to present different views and to make them comparable with earlier illustrations. Additionally, photos of free-floating genitalia were taken prior to final preparation to give an overview of its three-dimensional structure, as some diagnostic features are best viewed that way. Morphometrics of several genital features of *I. triglavensis* and *I. pirinella* sp. nov. are given in Suppl. material 1 (with two-directional T-tests applied when applicable). Numbers of measured samples (n) vary between species and sexes due to material limitations and in some instances also due to mounting artefacts resulting from three-dimensionality of genital structure and different preparation techniques. We therefore only included samples in the morphometric analyses that were comparable in terms of mounting and preparation technique.

**Photographic documentation.** Photographs of *I. triglavensis* specimens were taken with a Canon EOS 5D Mark III and Canon lens MP-E 65 at 2:1 using a ring flash. Genitalia photos were taken with a Wild Heerbrugg microscope using a 10× objective and a 2.5× ocular. Photos were edited using Helicon Focus 4.80 and Adobe Photoshop 6.0. For photography of free-floating genitalia, they were placed on a microscope slide with an excavation, filled with equal amounts of glycerol and water. A trace of anionic surfactant was added. Finally, the excavation was covered with a cover glass, leaving no air in the excavation. Photographs were taken with the same equipment as the embedded genitalia.

The camera system used for photos of adult *I. pirinella* was a Nikon D800 with Mikro Nikkor 105 mm 1:2.8 D objective and three flash heads. The camera was moved between shots with a Cognisys Stackshot focussing rail. Serene Stacker v. 1.04 and Adobe Lightroom 6.7 were used for processing the photos, and 36 shots were combined in each photo stack.

**DNA barcoding.** For the DNA analyses, tissue samples were sent in a lysis plate to the Canadian Centre for DNA barcoding, Ontario, Canada, through the Finnish Barcode of Life campaign ([www.finbol.org](http://www.finbol.org)). DNA extraction, amplification, and sequencing of the barcode region of the mitochondrial cytochrome oxidase I (COI) gene (658 base pairs at the 5' terminus) were carried out following protocols by deWaard *et al.* (2008). The taxonomic and collection data, voucher image, COI sequence and GenBank accession numbers are available for all specimens in the BOLD database (<http://www.boldsystems.org>) through the public dataset DS-INCURVEU ([dx.doi.org/10.5883/DS-INCURVEU](https://dx.doi.org/10.5883/DS-INCURVEU)).

We compared the DNA barcodes of *I. pirinella* with all other European species of *Incurvaria*, each of which had been barcoded along with national DNA barcoding initiatives in Finland, Austria and Germany. These data were supplemented with DNA barcodes of three other European species of the family Incurvariidae: *Alloclementia mesospilella* (Herrich-Schäffer, 1854), *Phylloporia bistrigella* (Haworth, 1828) and *Crinopteryx familiella* Peyerimhoff, 1871, the last

representing the subfamily Crinopteryginae (all other European species belong to the nominal subfamily). Therefore, only two European species of the family, *Paraclemensia cyanella* (Zeller, 1850) and *Vespina slovaciella* (Zagulajev & Tokár, 1990) are not included. Kimura 2-parameter (K2P) DNA barcode divergences were examined using the BOLD v. 4beta barcode gap analysis tool (Ratnasingham and Hebert 2007). A Neighbor-Joining (NJ) tree was constructed with Mega 7 (Kumar et al. 2016) under the Kimura 2-parameter nucleotide substitution model. A Maximum Likelihood (ML) analysis was conducted with Mega 7 under the GTR+G model for nucleotide substitutions. The node confidences were examined with 500 bootstrap replicates. Both trees were rooted on *Crinopteryx familiella*.

**Terminology.** The morphological terminology used here mainly follows Huemer (1993) and Davis (1998). In our comparisons of the newly described species with previously described species of the *vetulella*-group, we largely rely on the thorough revision by Huemer (1993).

## Results

The genus *Incurvaria* Haworth, 1828 contains nine described species in Europe (van Nieukerken and Kozlov 2017). Four of these species were assigned to the *Incurvaria vetulella* species-group in a revision by Huemer (1993): *I. vetulella* (Zetterstedt, 1839), *I. circulella* (Zetterstedt, 1839), *I. triglavensis* Hauser, 1912, *I. ploessli* Huemer, 1993. *Incurvaria pirinella* is a new member of European Incurvariidae clearly belonging to the *vetulella* species-group.

To the best of our knowledge, only four other species of *Incurvaria* are known from the Holarctic region: *I. evocata* (Meyrick, 1924) described from India, (Nielsen 1982) *I. takeuchii* Issiki, 1957 and *I.alniella* (Issiki, 1957) described from Japan (Okamoto and Hirowatari 2004) and *I. similella* Schmitz, 1969 described from the Caucasus (Schmitz 1969). These species do not belong to the *I. vetulella* species-group. In North America, only *I. vetulella* and *I. circulella* are known (Pohl et al. 2016, 2018).

*Incurvaria muchei* Soffner, 1969 from the Caucasus has been transferred to the genus *Alloclomensia* Nielsen, 1981 and considered as synonym of *A. devotella* (Rebel, 1893) (Nielsen 1981). The poorly known species *I. triangulifera* Tengström, 1869 has recently been considered to be a junior synonym of *Lampronia provectella* Heyden, 1865 (van Nieukerken and Kozlov 2017) and thus belongs to the family Prodoxidae. *Incurvaria kivatshella* Kutenkova, 1987, described from a single male specimen, was considered not to belong to the *vetulella*-group due to the absence of the distal projection of the phallus (Huemer 1993), but Kozlov (1996) synonymised it with *I. vetulella*. *Incurvaria brigantinella* Amsel, 1961 was described from Germany, Baden-Württemberg, Bodensee, based on a single type specimen, which is probably lost; it is therefore considered to be nomen dubium and was not included in Fauna Europaea (van Nieukerken and Kozlov 2017). The original description including a black and white drawing of the holotype clearly shows that *I. brigantinella* does not belong to the *vetulella* species-group. It resembles *I. praelatella* in having rather similar forewing markings and distinctly white forewing fringe.

## World checklist with type localities

*Incurvaria* Haworth, 1828

=*Excurvaria* Kuprijanov, 1994

*I.alniella* (Issiki, 1957). Japan, Honshu.

- I. circulella* (Zetterstedt, 1839). Sweden, Lappmark, Lycksele.  
(original combination *Adela circulella* Zetterstedt, 1839)
- I. evocata* (Meyrick, 1924). India, Assam.
- I. koernerella* (Zeller, 1839). Europe.  
(original combination *Tinea koernerella* Zeller, 1839)
- I. masculella* (Denis & Schiffermüller, 1775). Austria, Vienna.  
(original combination *Tinea masculella* Denis & Schiffermüller, 1775)
- I. oehlmanniella* (Hübner, 1796). Europe.  
(original combination *Tinea oehlmanniella* Hübner, 1796)
- I. pectinea* Haworth, 1828. Great Britain.  
(original combination *Tinea zinckenii* Zeller, 1839)
- I. pirinella* sp. nov. SW Bulgaria, Blagoevgrad, Pirin mountain range.
- I. ploessli* Huemer, 1993. Italy, Alps Maritime.
- I. praelatella* (Denis & Schiffermüller, 1775). Austria, Vienna.  
(original combination *Tinea praelatella* Denis & Schiffermüller, 1775)
- I. similella* Schmitz, 1969. Caucasus.
- I. takeuchii* Issiki, 1957. Japan, Honshu.
- I. triglavensis* Hauder, 1912. Slovenia, Vosshütte.
- I. vetulella* (Zetterstedt, 1839). Norway, Nordland.  
(original combination *Adela vetulella* Zetterstedt, 1839)  
=*Incurvaria kivatshella* Kutenkova, 1987

### Patterns of DNA barcode divergences of European Incurvariidae

Of five analysed specimens of *I. pirinella*, only one yielded a sequence (658 bp, full barcode fragment). Altogether 50 specimens of 13 species of European Incurvariidae, including all ten species of *Incurvaria*, were compared for their DNA barcodes. The species of the genera *Alloclementia*, *Phylloporia* and *Crinopteryx* (each containing only one species in Europe) show unique DNA barcodes (K2P divergences to the closest species 12.95–13.82%). For *Incurvaria* minimum K2P divergence to the closest species averages 7.05% (range 1.2–12.82%). The maximum intraspecific variation averages 0.81% across species (range 0–3.46%; mean  $n=4.2$ ; *I. pirinella* is not considered, as only one specimen was barcoded). The K2P distance between the genetically closest species exceeds 4.7% across all species except between *I. vetulella* and *I. ploessli*, which differ by a K2P divergence of 1.2% only. Moreover, *I. vetulella* appears paraphyletic with respect to *I. ploessli*, although the latter species forms a distinct barcode cluster.

### Description of the new species

#### *Incurvaria pirinella* Junnilainen, Kaitila & Mutanen, sp. nov.

<http://zoobank.org/DBDEC675-83A6-4AF6-A4C0-523D58C1E286>

**Type material.** Holotype: ♂, Bulgaria, Blagoevgrad district, Struma river valley, Stara Kresna 275m a.s.l., 41.795N, 23.157E; 03.v.2013. J. Junnilainen leg. & coll. with red label “HOLOTYPE of *Incurvaria pirinella* Junnilainen, Kaitila & Mutanen”. - Paratypes 21♂; 6♀: 4♂; 1♀ same locality and data as holotype, Genitalia prep. ♂ No: GPJJ201702, GPPB3334, 2♂ in glycerol, J. Junnilainen leg. & coll.; 7♂; 1♀ Bulgaria, Blagoevgrad district, southern Pirin 1200m a.s.l., 41.528N, 23.584E; 30.v.2006, Genitalia prep. ♂ No: GPJJ201701, 1♂ genitalia in glycerol, J. Junnilainen leg. & coll., 1♂ in. Tiroler Landesmuseum Ferdinandeum, Innsbruck, Austria.;



1♂ Bulgaria, Blagoevgrad district, southern Pirin 1300m a.s.l., 41.574N, 23.656E; 21–24.vi.2001 with green label DNA sample 24476 Lepid phyl., J. Junnilainen leg. & coll.; 4♂; 3♀ Bulgaria, Blagoevgrad district, Ilindenci road, meadows below barrier 900m a.s.l., 41.675N, 23.278E; 16.v.2012. J-P. Kaitila & Bo Wikström leg. coll. J-P. Kaitila, 1♂, 1♀ in Finnish Museum of Natural History, University of Helsinki, 1♀ in Tiroler Landesmuseum Ferdinandeum, Innsbruck, Austria. 2♀ Bulgaria, Blagoevgrad district, Slavianka, Goleshovo road, highest point 1000m a.s.l., 41.4034N, 23.389E; 25.v.2012, Genitalia prep. No: GPJJ201704. In coll. J-P. Kaitila; 5♂ Bulgaria, Blagoevgrad district, Ilindenci 900m a.s.l., 41.67N, 23.27E; 30.v.2012. Bo Wikström leg. & coll. Genitalia prep. No: GPJJ201701, GPJJ201702, GPJJ201704, GPPbf3327, GPPbf3328, GPPbm3329, GPPbm3330, GP-Pbm3334. All paratypes with red label “PARATYPE of *Incurvaria pirinella* Junnilainen, Kaitila & Mutanen”.

**Deposition of types.** The holotype of *Incurvaria pirinella* is deposited in the research collection of J. Junnilainen. Paratypes are deposited in the Finnish Museum of Natural History, University of Helsinki, Tiroler Landesmuseum Ferdinandeum (Innsbruck, Austria) and in the research collections of J. Junnilainen, J-P. Kaitila & Bo Wikström. The holotype is available on loan by request through the Finnish Museum of Natural History, University of Helsinki or directly from the first author.

**Etymology.** The name of the new species is derived from the Pirin mountain range, where the new species is widely distributed.

**Diagnosis.** Considering similar forewing ground colour and markings, *Incurvaria pirinella* (Figs 3–5) is externally closest to *I. circulella* (Fig. 14), but is easy to separate from it by the dark grey-brown fringe and distinct white spot in the forewing fringe present in *I. circulella*. *Incurvaria pirinella* is also widely allopatric as *I. circulella* occurs only in northern latitudes in Europe, e.g. the northern part of Sweden and Finland.

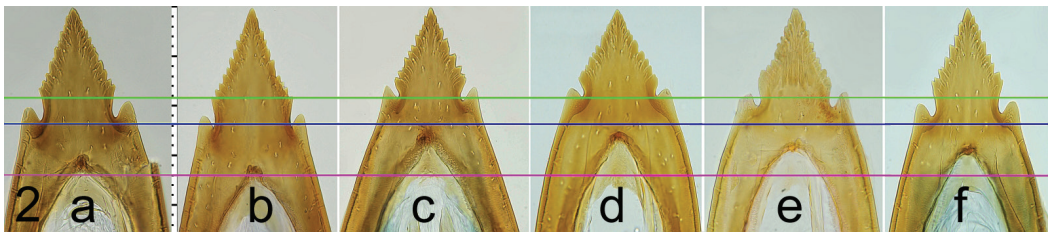
Although *I. triglavensis* is rather variable externally, it is always easy to separate from *I. pirinella* based on its relatively narrow forewing shape, paler ground colour and differences in forewing markings (Figs 6–11); see details in Suppl. material 2.

*Incurvaria pirinella* is easy to separate externally from Scandinavian *I. vetulella* (Figs 12–13) by a white narrow dorsal spot situated 2/5 from base and extending more than halfway across the forewing in *I. pirinella*. North European *I. vetulella* also typically have a distinctly broader dorsal spot, sparser forewing scaling and usually a pale medial area in the fringe, which is always absent in *I. pirinella*.

Male genitalia of *I. pirinella* are most similar to those of *I. triglavensis*, but small constant differences are present. *I. pirinella* has a stouter valva and vinculum. The valva of *I. pirinella* is broader in middle and the margin of the sacculus is more bulged (Figs 21–22). The vinculum of *I. pirinella* is relatively short and broad with clearly concave lateral margins. In *I. triglavensis*, the apex of the valva is more elongated and the vinculum is clearly longer and more slender with straight or slightly concave lateral margins (Figs 1a–b, 17e, 19b, 20e). The lateral arms of the transtilla are more robust (Fig. 20a), and submedial anterior projections shorter in *I. pirinella* (Figs 1c, 20d). The length of the transtilla’s medial knob with anterior submedial projection is 0.26–0.27 mm in *I. pirinella* and 0.29–0.33 mm in *I. triglavensis*. The juxta is longer (0.95–0.98 mm) in *I. pirinella* than in *I. triglavensis* (0.8–0.85 mm) (Fig. 16, Suppl. material 1). Also, the ratio between the length of the juxta versus the transtilla’s medial knob with its anterior projections is different between the species, being 3.56–3.77 in *I. pirinella* and 2.76–2.83 in *I. triglavensis* (see Suppl. material 1). The shape and angle of the distal thorn/hook-shaped projection of the phallus show variation, that is at least partly due to the position of the phallus in genitalia preparations (Figs 15d1, 18a–b); thus, this does not provide a good characteristic. However, the ratio between the maximum length



**Figure 1.** *Incurvaria pirinella*, green and *I. triglavensis* magenta. Shape of male genitalia compared: **1a.** left valva; **1b.** vinculum; **1c.** knob of transtilla with anterior projections.



**Figure 2.** *Incurvaria pirinella* and **c-f.** *I. triglavensis* length of female oviscap compared with coloured horizontal lines. Slide numbers **a.** GPPbf3327; **b.** GPPbf3328; **c.** GPPbf3324; **d.** GPPbf3325; **e.** GPPH-Inc17; **f.** GPPbf3335. Scale bar: 1 line = 0.01 mm.



**Figures 3–5.** *Incurvaria pirinella* habitus of adult. **3.** Male (Holotype). **4.** male (Paratype). Ilindenci road. Coll. Kaitila **5.** Female (Paratype). Slavianka. Coll. Kaitila.

of phallus versus the length of the distal projection seems to be different between the species as well (Figs 18a–b). In the examined samples, it was 3.34–3.78 for *I. pirinella* and 3.82–4.07 for *I. triglavensis*. Shape, size and ratio between length/width of abdominal segments T8 and S8 are different: See shape in Figs 23, 24, 25 and size and ratio measurement values in Suppl. material 1. Although *I. triglavensis* is a rather variable species, additional slightly different but sometimes



**Figures 6–11.** *Incurvaria triglavensis* habitus of adults. **6.** Male. Austria Osttirol-Deferegggen Gebirge 2200m 2013.vii.22 H. Deutsch leg.; **7.** Male. Slovenia Julijske Alpe Kamin, Sudseite 1700m 19.vii.1997 H. Deutsch leg.; **8.** Male. Slowenien-Steiner Alpe 1500m 1992.viii.12 Habeler leg.; **9.** Female. Slovenia, Steiner A. Krvavec Veli zwoh 20.vii.1992 1950m Habeler leg.; **10.** Female. Austria Salzburg Nockgebiet Matchan-shöhe 1900m 24.vi.1999 Habeler leg.; **11.** Female. Austria Steiermark Wöitzer Tauern Greimberg 1700m 26.vii.2001 Habeler leg. All in coll. TLMF.



**Figures 12, 13.** *Incurvaria vetulella* habitus of adults. **12.** Male. Finland EnL Enontekiö Tuolljehuhput 7676:8265, 13.-15.vii.2009 Marko Mutanen leg. & det.; **13.** Female. Finland EnL Enontekiö Saana 767:825, 16.vii.2012, Marko, Nestori & Anttoni Mutanen leg., coll. Marko & Tomi Mutanen.

overlapping features are found in other parts: details shown in Suppl. material 1. Genitalic structures of both sexes are overall more strongly sclerotised in *I. pirinella*.

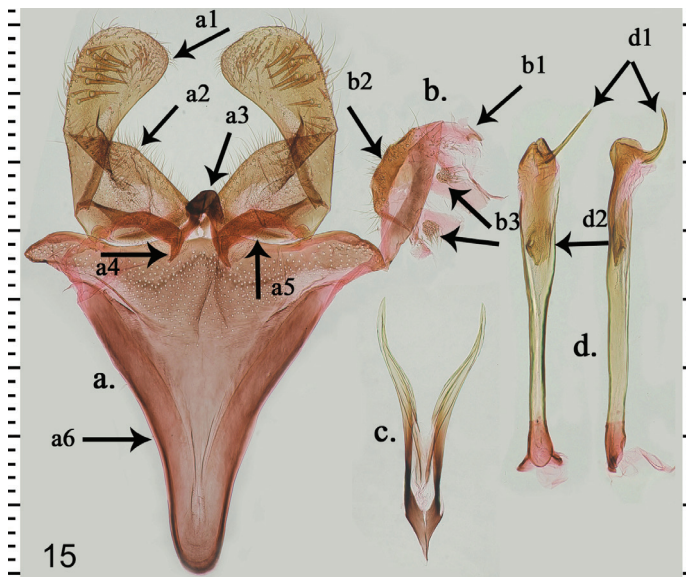
In the female genitalia, differences between *I. triglavensis* and *I. pirinella* are found in size of the oviscapt, especially distance from tip to bottom of basal excavation. In the examined material, it was 0.12 mm in *I. pirinella* and 0.09–0.11 mm in *I. triglavensis* (Fig. 2). The length of the apophyses posteriores is 2.70 mm in *I. pirinella* and 2.48–2.55 mm in *I. triglavensis*. Segment VIII also shows differences, but this feature shows overlap and therefore is not useful for determination of a single specimen. Differences between membranous parts of the bursa are not included as diagnostic characteristics, because they may show variation due to the age of specimens and may be altered after copulation. For more details, see Suppl. material 1 for morphometrics of genitalia of *I. triglavensis* and *I. pirinella* and photos of female genitalia (Figs 26–28, 30) of both species.

**Description.** Male (Figs 3–4). Wingspan 16.5–17.0 mm. Labial palp covered with brown and ochre-yellow scales, most pronounced on inner surface, 2<sup>nd</sup> segment equal in length to diameter





**Figure 14.** *Incurvaria circulella* male, Finland KiL. Muonio 7540:8362, 4.vii.2014, Marko, Anttoni & Nestori Mutanen leg., coll. Marko & Tomi Mutanen.

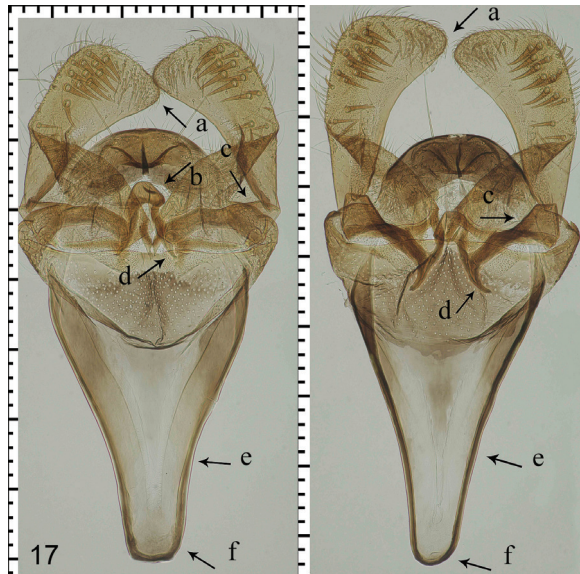


**Figure 15.** *Incurvaria pirinella* male genitalia unrolled: **a.** vinculum, transtilla and valvae; **a1.** apex of valva; **a2.** margin of sacculus; **a3.** medial knob of transtilla; **a4.** anterior submedial projection of transtilla; **a5.** strongly sclerotised lateral arm of transtilla; **a6.** strongly concave lateral margin of vinculum. **b.** Unrolled tegumen and uncus; **b1.** tegumen; **b2.** uncus; **b3.** socii. **c.** Juxta. **d.** Phallus from semilateral and lateral view; **d1.** hook-shaped distal projection of phallus; **d2.** sclerotised plate in vesica. Scale bar: 1 line = 0.05 mm.

of eye, pointed forward, 3<sup>rd</sup> segment half as long, turned upward. Antenna faintly ringed with dark brown and paler greyish-brown scales, slightly serrate, reaching three-quarters length of forewing. Head with yellow tufted scales. Thorax and tegula brown. Forewing relatively broad and roundish, ground colour grey-brown; two subtriangular yellowish-white dorsal spots, first narrower, 2/5 from base, extending over half width of forewing, second smaller, close to termen; a small yellowish-white triangular costal spot rather close to apex, sometimes a second usually smaller quadrangular yellowish-white spot close to the first one; fringe as ground colour. Hindwing and fringe fuscous. Abdomen and legs pale greyish-brown. Ventral side of forewing and fringe fuscous throughout except for distinct yellowish-white costal and dorsal markings.



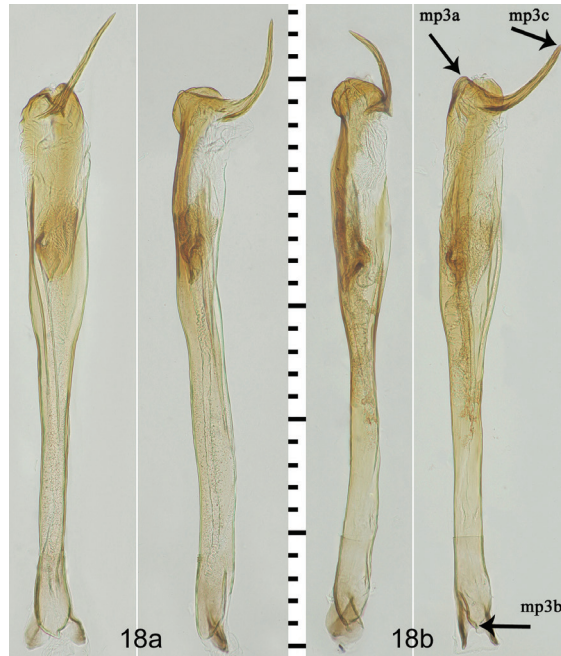
**Figure 16.** *Incurvaria pirinella* (left) and *I. triglavensis* (right) juxta: **a.** tip of lateral arm; **b.** medial part of lateral arm; **c.** basal part. Scale bar: 1 line = 0.05 mm.



**Figure 17.** *Incurvaria pirinella* left and *I. triglavensis* right male genitalia compared: **a.** apex of valva; **b.** medial knob of transtilla; **c.** subrectangular plate-like structure in lateral end of transtilla arm; **d.** anterior submedial projection of transtilla; **e.** lateral margin of vinculum; **f.** apex of vinculum. Scale bar: 1 line = 0.05 mm.

Female (Fig. 5). Wingspan 16.0–16.5 mm. Differs from male in darker grey-brown ground colour of forewings; two subapical costal spots always present; female dorsal spots larger and extending even more than 2/3 across forewing towards costa, the second white dorsal spot at tornus extending across the wing and sometimes reaching inner costal spot forming a complete fascia. Antennae not serrate.

**Male genitalia** (Figs 1, 15, 23, 25). Seven specimens examined. Uncus (Fig. 15b2) and tegumen (Fig. 15b1) short, forming together a relatively broad subrectangular plate, posterior margin of uncus somewhat roundish, medially and sublaterally slightly concave. Socii (Fig. 15b3) distinctly



**Figure 18.** a. *Incurvaria pirinella* and b. *I. triglavensis* phallus from semilateral and lateral view. mp3a-c. indicate measurement points parts of phallus. Scale bar: 1 line = 0.05 mm.

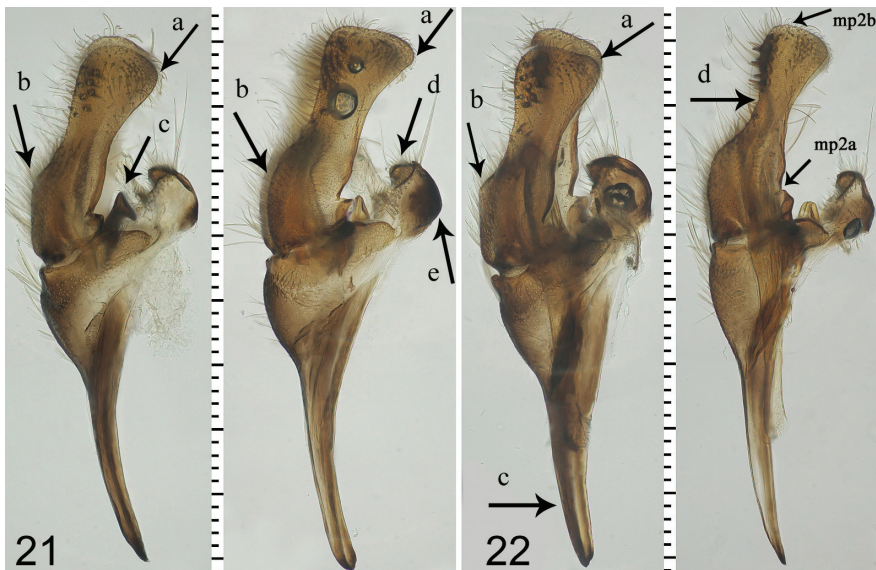
sclerotised, round-shaped and hirsute. Transtilla (Figs 15a3–a5) a conspicuous structure; medial knob (Fig. 15a3) and its lateral arms (Fig. 15a5) comparatively robust and strongly sclerotised; arms ending laterally to relatively low and broad subrectangular plate-like structure (Fig. 17c); submedial anterior projections (Fig. 15a4) digitate and relatively short. Valva relatively short and robust; costal margin comparatively slightly concave; dorsal margin of sacculus (Fig. 15a2) hirsute and somewhat obtuse-angled at 2/3 from base (Figs 21b, 22b); cucullus (Fig. 15a1) hirsute, moderately roundish with group of strong setae subdorsally and second smaller group of strong setae subdorsally near sacculus; apex hirsute and not significantly elongated (Fig. 21a). Vinculum V-shaped and relatively stout; lateral margins strongly concave (Fig. 15a6); posterior half strongly tapering towards slender distal half; apex moderately broad and roundish. Juxta (Figs 15c, 16) bifurcate, moderately long (length 0.95–0.98 mm), tip of lateral projections relatively pointed (Fig. 16a), medial part relatively narrow (Fig. 16b), basal part arrowhead-shaped and moderately strongly sclerotised (Fig 16c). Phallus (Fig. 15d) relatively long ( length without distal hook 1.21–1.27 mm); distally hook-shaped, also a relatively long (0.32–0.38 mm) strongly sclerotised projection (Figs 15d1, 18a-b), basal part broad plate-like, forming about 90° angle with hooked distal part; vesica with moderately short and broad irregular sclerotised plate at base (Fig. 15d2). Abdominal segments S8 and T8 (Figs 23, 25) as subrectangular plates. S8: narrow posterior margin slightly concave; wide anterior margin roundish; lateral margins slightly convex. T8: short and broad; posterior margin concave; anterior and lateral margins convex.

**Female genitalia** (Figs 2, 26–28, 30). Three specimens examined. Tip of oviscapt triangular, lateral margins concave, 9–11 small distinct teeth on both sides; basal notch somewhat U or V-shaped; lateral projection pointed and relatively short (Fig. 29), shape and size of lateral

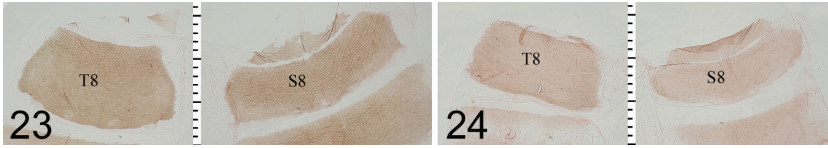




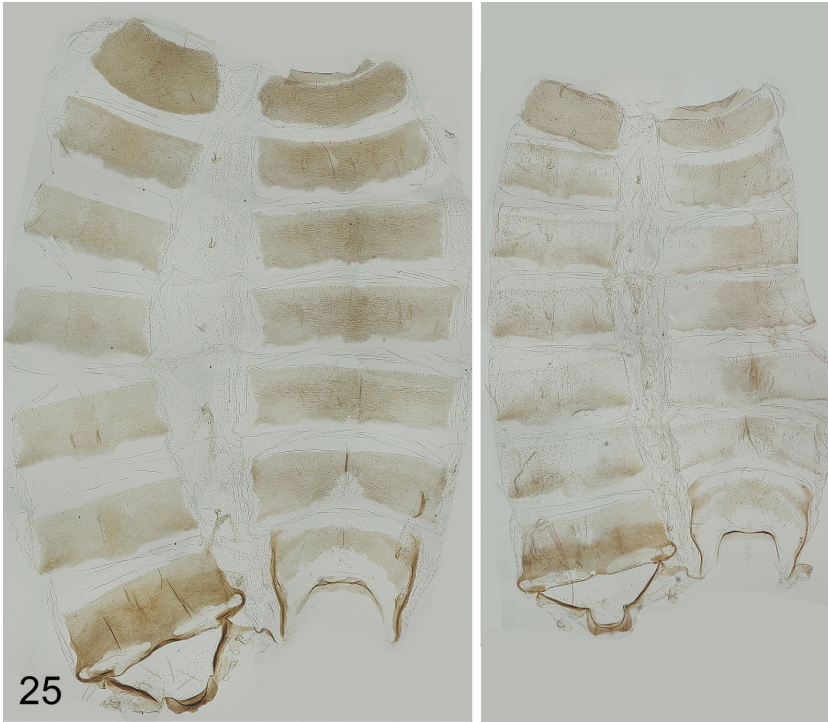
**Figures 19, 20.** Male genitalia from ventral view. **19.** *Incurvaria pirinella* left and *I. triglavensis* right: **a.** margin of uncus; **b.** lateral margin of vinculum. **20.** Male genitalia free-floating ventral view. *I. pirinella* left and *I. triglavensis* right: **a.** robust and strongly sclerotised lateral arms of transtilla; **b.** apex of valva; **c.** medial part of valva; **d.** medial knob of transtilla together with its anterior submedial projections; **e.** lateral margins of vinculum; **f.** apex of vinculum; **mp1a-b.** are measurement points length of vinculum. Scale bar: 1 line = 0.05 mm. Second smaller scale bar parallel to transtilla knob: 1 line = 0.01 mm.



**Figures 21, 22.** Male genitalia from lateral view. **21.** *Incurvaria pirinella* left and *I. triglavensis* right: **a.** apex of valva; **b.** sacculus; **c.** medial knob of transtilla; **d.** uncus; **e.** tegumen). **22.** Male genitalia free-floating lateral view. *I. pirinella* left and *I. triglavensis* right: **a.** apex of valva; **b.** sacculus; **c.** vinculum; **d.** medial part of valva; **mp2a-b.** are measurement points length of valva. Scale bar: 1 line = 0.05 mm.

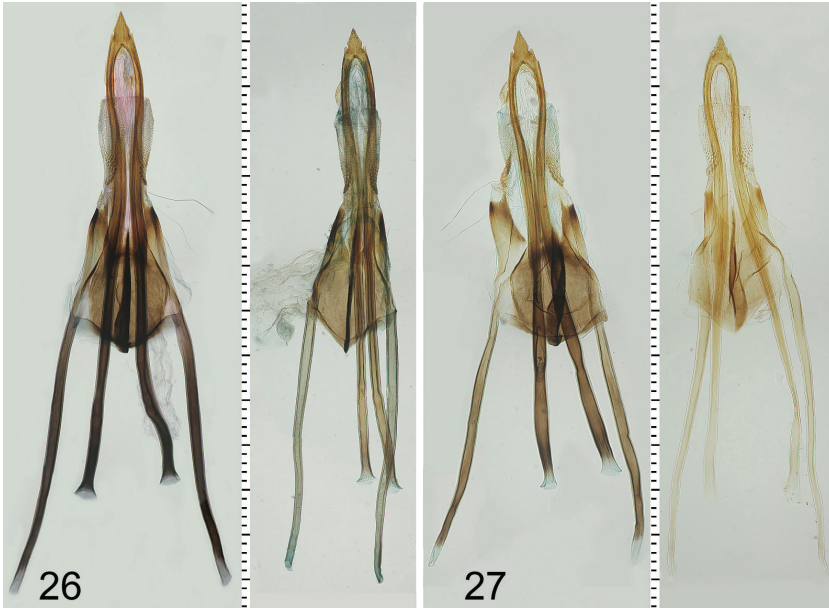


**Figures 23, 24.** Male abdominal tergites T8 and S8. **23.** *Incurvaria pirinella*; **24.** *I. triglavensis*. Scale bar: 1 line = 0.05 mm.

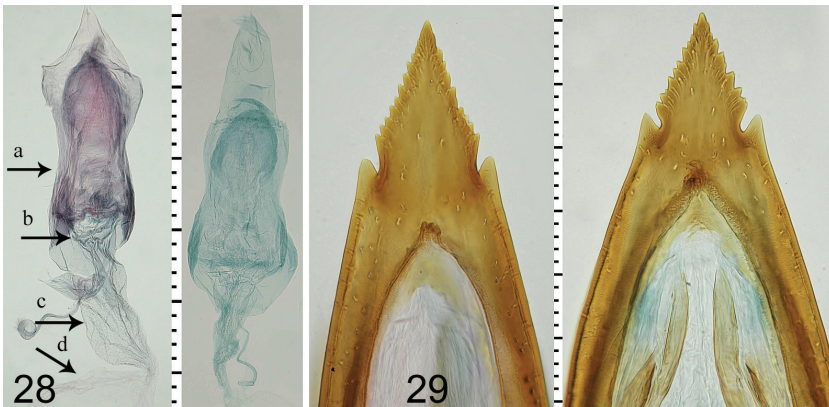


**Figure 25.** Male abdominal segments of *Incurvaria pirinella* (left) and *I. triglavensis* (right). Male abdominal segments.

margin teeth and basal notch variable. Apophyses relatively long (Figs 26–27, 30). Segment VIII a strongly sclerotised subrectangular plate (Figs 26a, 27); posterior 2/5 strongly tapering towards posterior end, anterior margin rounded. Vestibulum a subrectangular membranous sack (Fig. 28a), about twice as long as wide, posterior 1/3 angular, medially somewhat narrower, anterior half with rounded expansions on both lateral margins (expansions not clearly visible in photo because they are turned behind vestibulum), anteriorly membranous (Fig. 28b) funnel-shaped structure probably belonging to ductus bursa, 1/6 the length of remaining part of ductus bursae (Fig. 28c). Structure of ductus bursae complex, posterior 2/5 narrow tube-like, slightly widening anteriorly, anterior 3/5 broad sack-shaped, anterior end strongly tapered towards corpus bursae. Spermatheca spiral-shaped. Corpus bursae (Fig. 28d) a roundish sack, signa absent. Abdominal segments S8 and T8 relatively large subrectangular plates (Fig. 31). S8: posterior margin roundish and hirsute;



**Figures 26, 27.** 26. *Incurvaria pirinella* (left) and *I. triglavensis* (right). 27. *I. pirinella* (left) and *I. triglavensis* (right). Female genitalia from ventral view. Scale bar: 1 line = 0.05 mm.

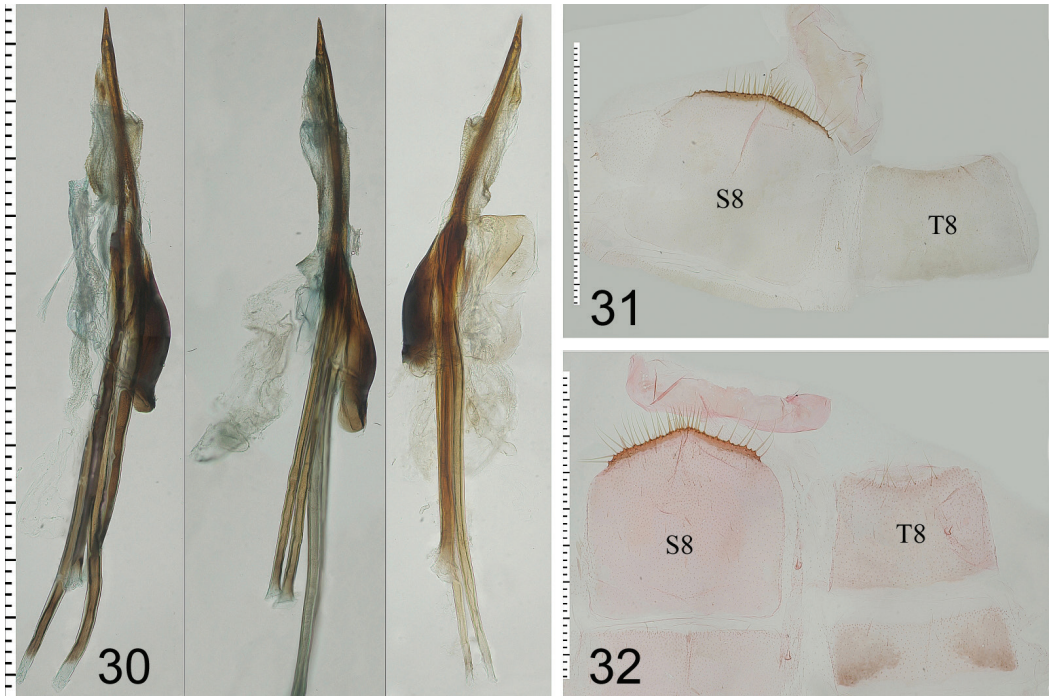


**Figures 28, 29.** 28. *Incurvaria pirinella* (left) and *I. triglavensis* (right) female bursae: **a.** vestibulum; **b.** membranous funnel-shaped structure in posterior end of ductus bursae; **c.** ductus bursae; **d.** corpus bursae. Scale bar: 1 line = 0.05 mm. 29. *I. pirinella* (left) and *I. triglavensis* (right). Oviscapt of female genitalia. Scale bar: 1 line = 0.01 mm.

anterior margin slightly concave; lateral margins almost straight. T8: posterior margin concave; broader anterior margin straight; lateral margins somewhat roundish.

**Molecular diagnostics** (Fig. 33). Barcode Index Number (BIN, see Ratnasingham and Hebert 2013): BOLD:ACW2589. The nearest neighbour of *I. pirinella* in our dataset is *I. triglavensis* (BOLD:AAI8097) with a K2P minimum divergence of 4.74%. The next closest species are *I. vetulella* (BOLD:AAD4279)





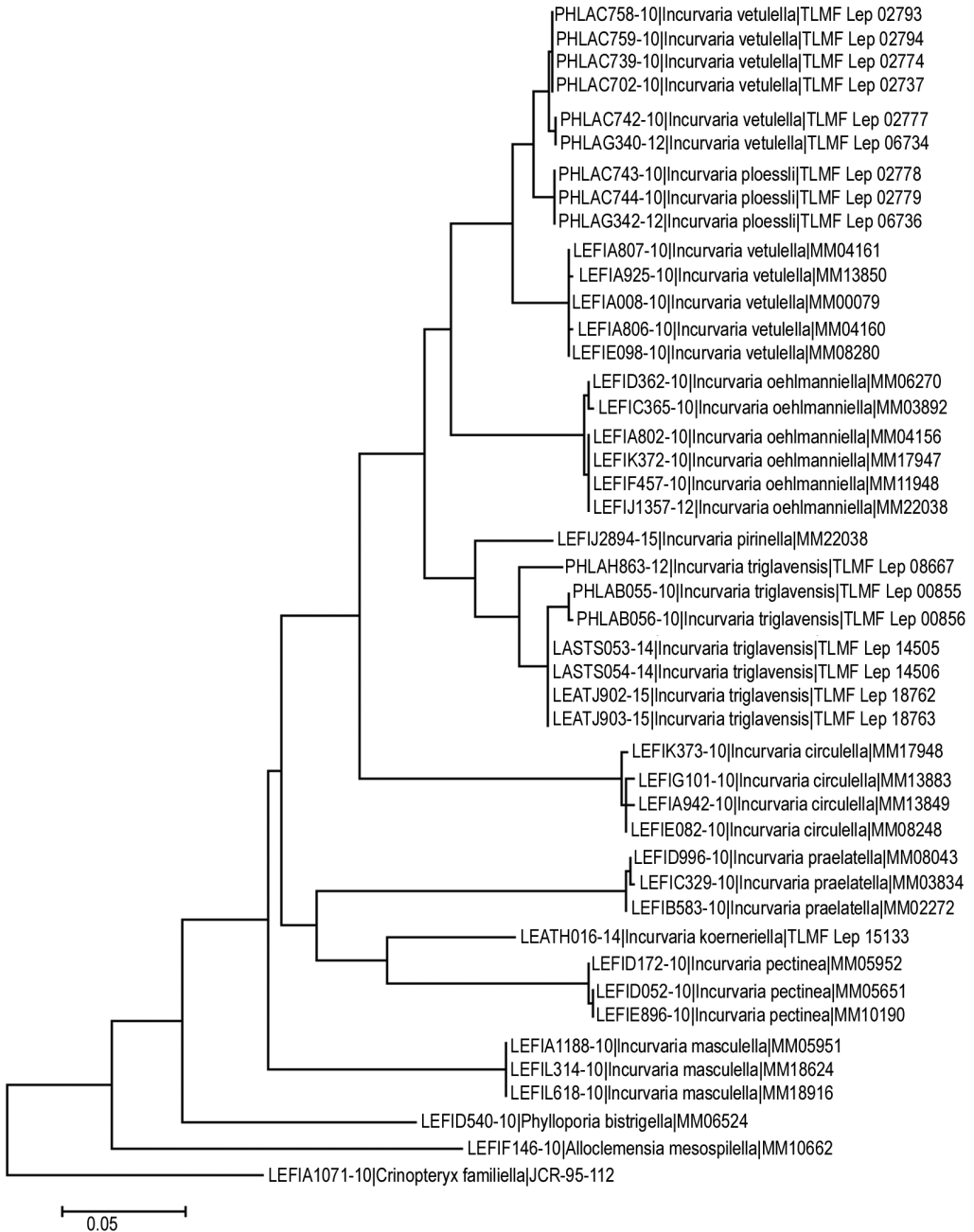
**Figures 30–32.** 30. a. *Incurvaria pirinella*; b. and c. *I. triglavensis*. Female genitalia free-floating from lateral view. Scale bar: 1 line = 0.05 mm. 31. *Incurvaria pirinella*; 32. *I. triglavensis*. Female abdominal tergites S8 and T8. Scale bar: 1 line = 0.05 mm.

with 7.06%, *I. ploessli* (BOLD:AAP5641) with 7.07% and *I. oehlmanniella* (BOLD:AAD1334) with 8.45% minimum divergences, respectively.

**Variation.** The external habitus of both sexes is rather constant. Females are darker overall than males. One male has a second yellowish white costal spot near forewing apex. Sometimes the tornal spot and inner subapical costal spot form a complete fascia in the female forewing. Genitalic structures of both sexes are only slightly variable, whereas those of *I. triglavensis* show significant variation both externally and in genitalic structures of both sexes.

**Distribution.** Known from four different localities in the south-west corner of Bulgaria around the Struma river valley and its adjacent regions, which all belong to the Blagoevgrad district and the Pirin mountain range. The elevational range is wide: 200–1200 m, at least.

**Biology.** Many specimens were captured with insect nets during daytime, and females especially were disturbed in the evening from shrubs such as *Rosa* L. Males were observed swarming early in the morning, but they were also caught with artificial light late at night, which is an unusual collecting method for the species of the *vetulella*-group in the Alps (P. Huemer pers. comm.) and northern Europe (own observations, and although summer nights are light in the North, *I. circulella* comes readily to light). *Incurvaria pirinella* is an early species, flying at lower elevations in early May and at higher elevations in late May and June. The biology of the early stages



**Figure 33.** Maximum likelihood tree as based on DNA barcodes (658 bp fragment of the mt COI gene) of European *Incurvaria* species and three members of other genera of Incurvariidae. The node support values are based on 500 bootstrap replicates. The tree was rooted on *Crinopteryx familiella* (Crinopteryginae).



**Figure 34.** Habitat of *Incurvaria pirinella* Slavianka, Southern Pirin 1000 m a.s.l.

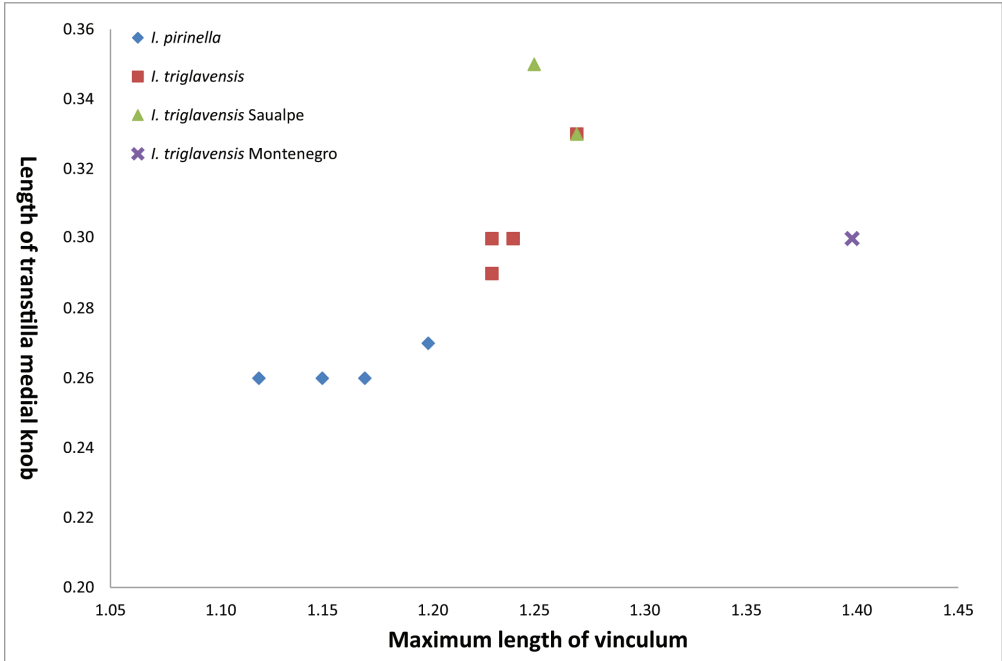
remains unknown. The food-plant of *I. vetulella* is reported to be *Vaccinium* L. (Ericaceae), especially *V. myrtillus* L. (Klimesch 1961, Wojtusiak 1976), but the life history details for the other members of the *vetulella*-group remain largely unknown. It is suspected that the other European members of the group are also associated with *Vaccinium* (Huemer 1993). However, no species of *Vaccinium* or *Erica* L. are present, at least not in most localities where we have found *I. pirinella*, suggesting that they are not its food-plants. Specimens of *I. pirinella* have been collected from completely different habitats from the other species of the *vetulella*-group, which in the Alps are always found in alpine areas usually above timberline and especially from north-facing slopes. In Scandinavia, *I. vetulella* occurs in alpine and subalpine habitats, but also boreal coniferous forest and boggy habitats. *Incurvaria pirinella* seems to prefer open xerothermic south-facing slopes with rich deciduous forest edges of *Quercus* L., and *Fagus* L. (Fig. 34), and a variety of shrubs, including *Crataegus* L., *Corylus* L., and *Rosa*, sometimes also *Cornus* L., *Malus* Mill., *Pyrus* L., and *Chamaecytisus* Link. Along with *I. pirinella*, *I. masculella*, well known as an early flying species, was collected in numbers.

## Discussion

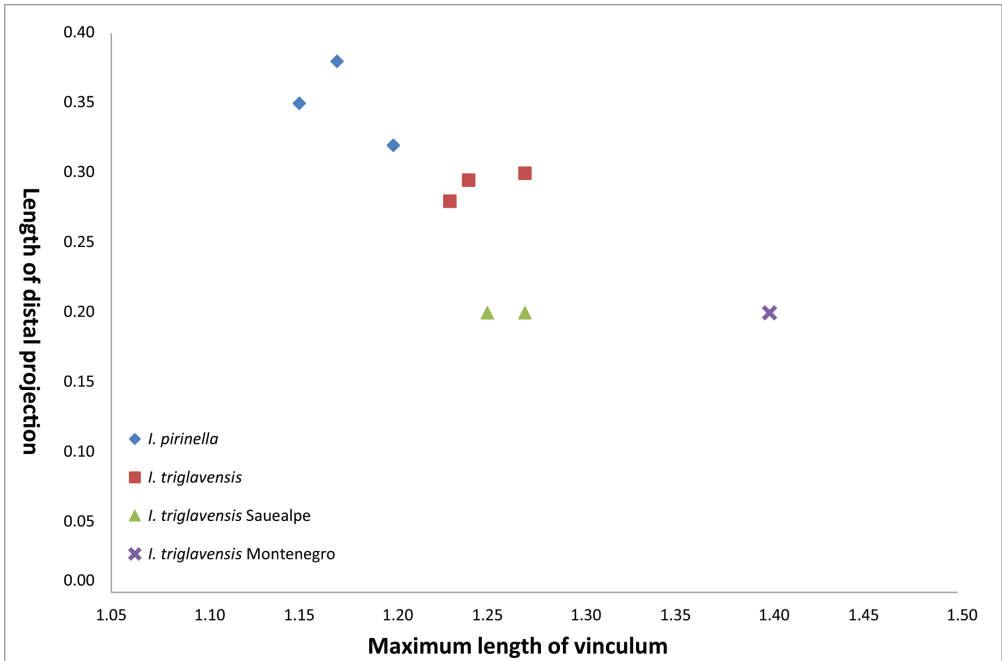
To our knowledge, detailed illustrations of female genitalia of *Incurvaria* have not been published before. Only the oviscapt plate was illustrated in earlier publications on the *Incurvaria vetulella* group



35



36



Figures 35, 36. Morphometrics of *Incurvaria pirinella* and *I. triglavensis* with genetically different populations from Saualpe and Montenegro of the latter species included. 35. Length of vinculum versus transtilla medial knob with anterior projections. 36. Length of vinculum versus distal projection of phallus. The measurements are in millimetres.

(Laasonen *et al.* 1981, Huemer 1993). The size and the shape of the oviscapt appear useful new characteristics, but the number and shape of lateral teeth of oviscapt seems to vary quite a lot even within species. We found also significant differences in the length of apophyses posteriores and minor differences in the shape and length of segment VIII and the shape and size of abdominal plates T8 and S8.

DNA barcodes of *I. vetulella* fall into two clearly separate clusters within Europe, with *I. ploessli* being nested within these clusters, rendering *I. vetulella* paraphyletic. The first group contains samples from North European countries and the second samples from the Alps. We suspect that the *I. vetulella* population in the Alps actually represents an undescribed taxon, as *I. vetulella* shows an overall 3.46% intraspecific divergence in DNA barcodes in Europe. Genomic-based studies on whether these clusters represent a different species are underway (Huemer and Mutanen *in prep.*). In Central Europe, the *vetulella*-group contains also *I. triglavensis* and *I. ploessli*. We consider *I. pirinella* as a new member of this species group. Each of the Central European species appears to have a rather restricted distribution. Although *I. triglavensis* has also been mentioned from Balkan countries such as Bosnia and Herzegovina (Huemer 1993), further material is needed to clarify the status of the Balkan specimens, because the single barcoded specimen from Montenegro deviates significantly from other barcoded samples of *I. triglavensis*. Moreover, a new DNA barcode cluster was recently found from the Saualpen in Austria by Huemer (personal communication). These new findings indicate that the *I. vetulella*-group should be revised again.

Originally, genetically deviant specimens of *I. triglavensis* from the Alps and Montenegro were included in the morphometric analyses. While some morphological differences between them and other *I. triglavensis* as well as *I. pirinella* were observed, we excluded them from the final analyses (see Figs 35–36) as this was beyond the scope of our study. The illustrated *I. triglavensis* samples were verified not to include specimens of this putatively undescribed species.

In northern Europe, the superficially similar *I. circulella* occurs sympatrically with *I. vetulella*. Despite superficial resemblance of these species, Huemer (1993) considered that *I. circulella* does not belong to the *vetulella*-group. This is supported by the maximum Likelihood analysis of DNA barcodes, as *I. circulella* appears also genetically relatively distant to the species of the *vetulella*-group. It forms a sister lineage to the *vetulella*-group + *I. oehlmanniella*. Based on the DNA barcodes, *I. oehlmanniella* is nested within the *vetulella*-group (Fig. 33). However, a phylogenetic analysis based on DNA barcodes alone may be misleading due to a single locus and due to the small number of characters, and further studies, preferably including nuclear markers, are needed. Interestingly, Bengtsson *et al.* (2008) consider *I. circulella* as likely to feed on *Betula* spp., which we find unlikely as this species is often abundant in spruce “taiga” forests with plenty of *Vaccinium myrtillus* but no or little *Betula* L. (Betulaceae). Misleading habitat and food plant association may be the reason why Bengtsson *et al.* (2008) consider *I. circulella* a rare species. In Finnish Lapland it is common, especially in spruce forest habitats (“taiga” forests), but does not occur or is rare in the subalpine and alpine habitats where *I. vetulella* is often abundant. The males of both *I. circulella* and *I. vetulella* swarm most actively late at night and in the early morning.

In our Maximum likelihood analysis (Fig. 33), *Incurvaria* appears to be monophyletic, although with poor support as the distances between the species are generally large. Also, several of its basal branches are short and groupings are overall poorly supported. The *vetulella*-group together with *I. oehlmanniella* is well supported.

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Kimmo Silvonen (Finland) took and processed the photos of the adults and Pasi Sihvonen and Bo Wikström (Finland) took and processed the photos of genitalia. Lauri Kaila (Finland) provided valuable comments and information concerning our manuscript. Martin Corley (United Kingdom) improved our English of an earlier draft of the manuscript. Peter Huemer (Austria), kindly provided his DNA barcode records of European *Incurvaria*, and specimens of *Incurvaria* for morphological examination. We thank him also for many valuable discussions on *Incurvaria* taxonomy. Andreas Segerer (Germany), gave us a permission to include his DNA barcode record of *I. koerneriella*. Mikhail Kozlov (Finland), Peter Huemer, David Lees (UK) and Erik J. van Nieukerken (The Netherlands) provided many helpful comments on earlier versions of the draft. We are grateful to all of these mentioned people for their valuable help. We are indebted to the staff of the Canadian Centre for DNA Barcoding for their continuous help with management of DNA barcode data. Finally, we thank the Lepidopterological Society of Finland, Kone foundation, Finnish Cultural foundation and the Academy of Finland (#283609) for financial support for DNA barcoding of samples.

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## Supplementary material 1

### **Morphometrics of genitalia of *I. triglavensis* and *I. pirinella* sp. nov.**

Authors: Jari Junnilainen, Peter Buchner, Jari-Pekka Kaitila, Marko Mutanen

Data type: measurements

Explanation note: The measurements are in millimetres.

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Link: <https://doi.org/10.3897/nl.42.13026.suppl1>

## Supplementary material 2

### **External characteristics of the European *Incurvaria vetulella* species-group**

Authors: Jari Junnilainen, Peter Buchner, Jari-Pekka Kaitila, Marko Mutanen

Data type: species data

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