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A non-destructive virtual dissection by micro-CT reveals diagnostic characters in the type specimen of *Caloptilia stigmatella* (Lepidoptera: Gracillariidae)

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Abstract

Nearly a century ago, wing venation was introduced in gracillariid taxonomy as a means to diagnose closely related genera and species groups. Recent advances in non-destructive virtual micro-dissections suggest promising approaches with which to revisit the relevance of wing venation characters on historic primary type specimens. Many unique type specimens in Gracillariidae and other microlepidoptera groups preserved in museum collections are in poor condition, and over the course of history have suffered loss or damage to their abdomens. Consequently, genitalia morphology is not available for diagnoses and comparisons. In this paper we emphasize the need to include the type species and type specimens into the broader context of taxonomic studies on micro-moths in general and the family Gracillariidae in particular. The genus *Caloptilia* has a world-wide distribution and has been the subject of research for more than 200 years, yet the generic boundaries and groupings within the genus are still unresolved due to the lack of a reliable set of taxonomic characters obtained from the primary types. We describe a method of virtual descaling of the fore- and hindwings using the unset micro-moth type specimen of *Caloptilia stigmatella* Fabricius, 1781, in order to demonstrate that the study of historic and fragile type specimens and diagnoses of their internal morphological characters becomes possible by applying new and non-destructive technology.

Key words: *ampelipennella*, computed tomography, genus-level diagnosis, holotype, nomen nudum, non-destructive dissection, visual method

Introduction

Following the rules of the ICZN (1999), species names are based on actual specimens which are represented by primary types (holotypes, lectotypes, syntypes) deposited in public repositories. The existence of such type specimens and the physical embodiment of the species names increases the precision of available diagnostic characters observed in the type specimens and minimizes identification errors. Genera, being the conventional units, are based on the same principal: every genus-group name is eternally linked to the type species designated by an author (original designation), by a reviser (subsequent designation), or based on monotypy. The conventional generic concept in micro-moths, a typically pragmatic approach based on limited morphological data, must now be reconciled with new molecular and morphological data sets to shed light on the phylogeny and taxonomy of a particular group (e.g., Mutanen *et al.* 2010; Kawahara *et al.* 2011, 2017; Regier *et al*. 2013). The search for conventional generic concepts in the family Gracillariidae and particularly revising the species-rich genus *Caloptilia* Hübner, 1825 by the community of taxonomists aims to focus on long-term taxonomic solutions and seeks to group species into meaningful sets. In this sense the application of advanced imaging-computed technology may play a useful role as a tool efficiently mediating between different hypotheses (e.g., Kitching & Simonsen 2014; Leubner *et al*. 2017).

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One of the morphological datasets that delimits genera within Gracillariidae is wing venation (Ely, 1918). The generic stability of this character set and its application to define the known genera and to describe new ones has been demonstrated by Vári (1961) and Kuznetzov (1981). However, the majority of the genera of Gracillariidae were erected without taking wing venation into account, and this information has not been corroborated with the type specimens. The traditional study of wing venation, which includes physical descaling the wings, is highly invasive. It is not applicable for unspread specimens because the risk of destroying such specimens is too high (Zimmerman 1978). For this reason we looked for a technique that allows taxonomists to examine wing venation characters while keeping valuable type specimens intact.

Micro-computed tomography (μ-CT) has been shown to be a useful tool in the study of insect morphology. It has been used successfully for virtual dissections of the genitalia of macrolepidoptera. Such visualizations have allowed individual components of complex structures to be observed in isolation with minimal interference from other components without physically separating them, proving much less destructive than real dissection (Simonsen & Kitching 2014; Saito *et al*. 2017). The use of μ-CT has been advocated for taxonomic studies where a researcher needs to compare already dissected specimens with types to determine their identity. Simonsen & Kitching (2014) highlight the particular value of this approach for invaluable legacy collections (e.g. the Linnaean and Fabricius collections). Hence, we decided to apply this technique to study the holotype of *Caloptilia stigmatella* (Fabricius, 1781).

Caloptilia is the third largest genus in the family Gracillariidae after *Phyllonorycter* Hübner, 1822 and *Acrocercops* Wallengren, 1881, and it has a global distribution (De Prins *et al*. 2015; De Prins & De Prins 2018). The leaf-mining moth *C. stigmatella* (Accession number: GLAHM 137070) was described by Fabricius in 1781 from an English specimen in the collection of Thomas Pattison Yeats (Stainton 1864). It has been in William Hunter's insect collection since Yeats died in 1782 (Hancock 2015) and is kept at the Hunterian Museum, University of Glasgow. In addition to being the type specimen of the species, it is also the type species for the genus *Caloptilia* (Vári 1961; Kumata 1982; De Prins & De Prins 2005, 2018). The specimen is generally in good condition but is not ideal for morphological examination as it is mounted with its wings closed and at some point in history its abdomen has been lost.

The purpose of the present paper is: (1) to examine this valuable Fabrician specimen by virtual descaling using the μ-CT scanning technique; and (2) to evaluate this non-destructive methodology for revealing the diagnostic characters of taxonomic significance in fragile, historically important specimens.

Taxonomic data acquisition

Historical designation of *Tinea stigmatella* as the type species of the genus *Caloptilia*

The original description of the *Caloptilia* by Hübner in 1825 as "Verein, Coitus I" in the group of moths "Bemercte, Signatae" indicates that the main diagnostic character is the colouration of the forewings: "Mit hellen Zeichen auf den Schwingen, in färbigen Grunde." This feature of colourful forewings marked by different signs distinguishes *Caloptilia sensu* Hübner from other micor-moths with dull greyish-brownish forewings mainly under the name *Tinea*. Hübner (1825) listed two species under the Coitus I [the category in present understanding refers to a genus]:

4160. *Caloptilia upupaepennella* (Hübner, 1796), originally described as *Tinea upupaepennella* in: Hübner, J. 1796–1838. Sammlung europäischer Schmetterlinge. Achte Horde. Tineae Die Schaben; nach der Natur geordnet, beschrieben und vorgestellt: 68, pl. 30, fig. 203;

4161. *Caloptilia ampelipennella* (Hübner, 1825), originally described as *Tinea hybnerella* in: Hübner, J. 1796–1838. Sammlung europäischer Schmetterlinge. Achte Horde. Tineae Die Schaben; nach der Natur geordnet, beschrieben und vorgestellt: pl. 34, fig. 236.

C. ampelipennella is a nomen nudum [fails to conform to Article 12.1 of the ICZN].

Tinea upupaepennella Hübner, 1796, with the type locality of Augsburg, Germany, became the type species of the genus *Caloptilia* Hübner, 1825 by subsequent designation by Fletcher (1929). However, *Tinea upupaepennella*

Hübner, 1796 became a junior subjective synonym of *Tinea stigmatella* Fabricius, 1781, synonymized by Haworth (1828: 529). Fabricius was the first to present a description of this species, as *Tinea stigmatella* in 1781, based on a specimen that was collected in England, United Kingdom. The description conforms to ICZN Article 12, and agrees with the principle of typification. The specimen was re-examined, and we found that the characters fully correspond with the description of Fabricius (Figs 1, 2). The accompanying documentation and labels stating the identity and the taxonomic status of the specimen were provided. The species-group name *Caloptilia stigmatella* comprises ten synonymic nominal species-group names (Table 1). The re-verification of the full synonymy of *C. stigmatella* (Table 1) based on the re-study of type specimens of each species-group name is outside the scope of the present paper.

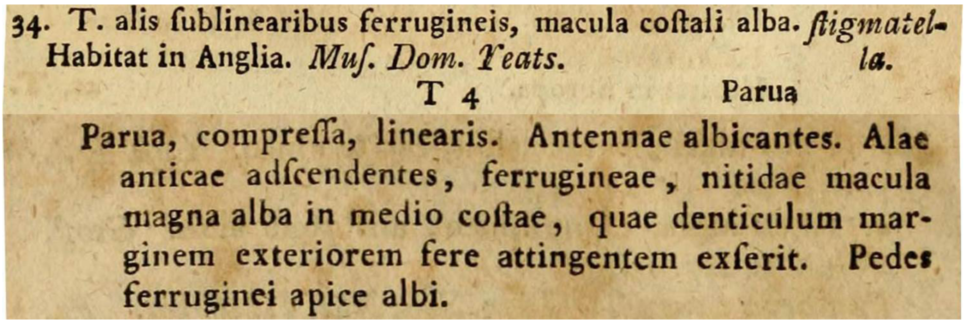
34. T. alis ſublinearibus ferrugineis, macula coſtali alba. *ſtigmatella.*
Habitat in Anglia. *Muſ. Dom. Yeats.*
T 4 Parua
Parua, compreſſa, linearis. Antennae albicantes. Alae anticae adſcendentes, ferrugineae, nitidae macula magna alba in medio coſtae, quae denticulum marginem exteriorem fere attingentem exſerit. Pedes ferruginei apice albi.

FIGURE 1. Original material. The original description of *Tinea stigmatella* by Fabricius (1781). The description is under the number 34.

FIGURE 2. Original material. 2. Type specimen of *Tinea stigmatella* (Fabricius, 1781) GLAHM:137070 kept at the Hunterian, University of Glasgow. The handwritten number 34 on the label matches the number in original description by Fabricius.

TABLE 1. Synonymy and taxonomic acts of the *Caloptilia stigmatella.*

Species-group name	Type locality	Taxonomic act
[*Phalaena*] [*Tinea*] *cruciella* Goeze, 1783	[Germany]	A junior subjective synonym of *Tinea stigmatella* Fabricius, 1781
T.[*inea*] *equestris* de Fourcroy, 1785	[France], Paris	A junior subjective synonym of *Tinea stigmatella* Fabricius, 1781
Tin.[*ea*] *upupaepennella* Hübner, 1796	[Germany], [Augsburg]	A junior subjective synonym of *Tinea stigmatella* Fabricius, 1781, synonymized by Haworth (1828: 529)
Tinea triangulella Panzer, 1794	[Germany], Dresden	A junior subjective synonym of *Tinea stigmatella* Fabricius, 1781, synonymized by Duponchel (1838–1840: 602)
G.[*racillaria*] *purpurea* Haworth, 1828	[United Kingdom], London	A junior subjective synonym of *Tinea stigmatella* Fabricius, 1781, synonymized by Stainton (1849: 22)
G.[*racillaria*] *ochracea* Haworth, 1828	[United Kingdom], London	A junior subjective synonym of *Tinea stigmatella* Fabricius, 1781, synonymized by Stainton (1849: 22)
G.[*racillaria*] *trigona* Haworth, 1828	[Germany], Dresden	An unjustified emendation of *Tinea triangulella* Panzer, 1799, nec *Tinea trigonella* Linnaeus, 1758 and a junior subjective synonym of *Tinea stigmatella* Fabricius, 1781, synonymized by Stainton (1849: 22)
Tinea (*Pl.*[*utella*]) *triangolosella*; *Pl. triangulosella* Costa, 1836	[Italy], Naples	A junior subjective synonym of *Tinea stigmatella* Fabricius, 1781, synonymized by Heydenreich (1851: 90).
Gracillaria purpuriella Chambers, 1872	[United States], [Kentucky]	A junior subjective synonym of *Tinea stigmatella* Fabricius, 1781, synonymized by Chambers (1879: 74)
G.[*racilaria*] *consimilella* Frey & Boll, 1876	[United States], Texas, Dallas	A junior subjective synonym of *Tinea stigmatella* Fabricius, 1781

Taxonomic value of the type specimen *Caloptilia stigmatella*

The specimen collected in England and described by Fabricius as *Tinea stigmatella* from the Thomas Pattinson Yeats collection became a reference not only for the species-group name *stigmatella* (Table 1), but also for one of the most speciose genera in leaf-mining micro moths. *Caloptilia* contains 474 species-group names of which 323 species are valid (De Prins & De Prins 2018). This is why the discovery of this specimen in the Hunterian Museum, University of Glasgow is so significant. The wide distribution of the species (Table 2), and more than 53 host-plant species recorded for *C. stigmatella* (De Prins & De Prins 2018), the slight variability in morphological characters of wing venation and male genitalia, and six molecular haplotypes (BOLD 2018) all suggest that the nominal taxon *C. stigmatella* might comprise a complex of species-group taxa.

Although the objective of the present paper is not to delimit *C. stigmatella* as a species-group taxon, characters of wing venation observed in the holotype will allow us to match and diagnose the holotype with other specimens of different populations in which variability and different character states are found, such as the coalescence of R_4 and R_5, M_2 and M_3. Slight variation in venation concerns whether the bases of R_4 and R_5 distal to the discoidal cell are connate or separate, or whether the bases of M_1 and M_2 distal to the discoidal cell are short stalked or connate. This slight variation still needs to be evaluated in order to define whether it represents intra- or inter-specific variation (Albrecht & Kaila 1997). Also, the delineation of the genus *Caloptilia* is not solved yet and currently is under investigation (Arévalo-Maldonado in prep.). There were attempts in the course of history to group *Caloptilia*-like species and to diagnose the genus *Caloptilia*, especially with the morphologically very similar genus *Gracillaria* Haworth, 1828 (De Prins 1985), which is the type genus of the family.

In the present paper we attempt to fine-tune the diagnosis of the genus *Caloptilia*, referring to the direct evidence—the primary type specimen [holotype] examined and described by Fabricius 237 years ago.

TABLE 2. Distribution records of *Caloptilia stigmatella.*

Country	Reference of the first record
Palaearctic	
Armenia	Kuznetzov, V. I. 1981: 182
Austria	Duponchel, P. A. J. 1845: 372
Belgium	De Sélys-Longchamps, E. 1844: 25
Bosnia and Herzegovina	Rebel, H. 1904: 368
China	Kuznetzov, V. I. 1981: 182
Croatia	Rebel, H. 1904: 368
Czech Republic	Duponchel, P. A. J. 1845: 372
Denmark	Bang-Haas, A. 1875: 36
Estonia	Frey, H. 1856: 230
Finland	Tengström, J. M. J. 1848: 144
France	Duponchel, P. A. J. 1838–1840: 604
France: Corsica	Skala, H. 1938: 44
Georgia	Kuznetzov, V. I. 1981: 182
Germany (as *upupaepennella*)	Hübner, J. 1796–1838: 68
Germany	Biesenbaum, W. 2016: 43
Hungary	Szöcs, J. 1981: 217
Ireland	Meyrick, E. 1927a: 791
Italy (as *triangulosella*)	Costa, O. G. 1836: 2–3
Italy	Glerean, P. & Triberti, P. 2015: 63
Italy: Sardinia	De Prins,W. & De Prins, J. 2005
Italy: Sicily	Massa, B., Rizzo, M. C. & Caleca, V. 2001: 93
Japan: Hokkaido, Honshū	Kunata, T. 1982: 34
Korea, Republic of	Park, K. T. 1983: 62
Kyrgyzstan	Kuznetzov, V. I. 1981: 182
Latvia	Lienig, F. 1846: 297
Liechtenstein	De Prins, W. & De Prins, J. 2005: 127
Lithuania	Ivinskis, P. & Pakalniškis, S. 1984: 27
Luxembourg	Wagner-Rollinger, C. 1974: 307
Macedonia	De Prins, J. & De Prins, W. 2017
Mongolia	Kuznetzov, V. I. 1981: 182
Morocco	Rungs, C. E. E. 1979: 49
Netherlands	de Graaf, H. W. 1853: 48
Norway	Strand, E. 1901:40
Poland	Büttner, F. O. 1880: 451
Portugal	Mendes D'Azevedo, C. 1913: 25
Romania	Caradja, A. 1899: 208
Russian Federation: Central Asia and Siberia	Martynova, E. F. 1952: 86
Russian Federation: European Part	Zeller, P. C. 1847: 322
Russian Federation: Far East	Ermolaev, V. P. 1977: 108
Russian Federation: Far East (Khabarovsk Region, Kuril Islands, Primorye, Sakhalin)	Noreika, R. 1997: 392

......continued on the next page

TABLE 2. (Continued)

Country	Reference of the first record
Slovakia	Skala, H. 1937: 11
Spain	De Prins, W. & De Prins, J. 2005: 127
Sweden	Zeller, P. C. 1847: 322
Switzerland	Frey, H. 1856: 231
Tajikistan	Sherniyazova, R. M. 1975: 188
Turkey	Koçak, A. Ö. & Seven, S. 2001: 1
Turkmenistan	Rebel, H. 1904: 368
Ukraine	Gershenzon, Z. S. & Kholtshenkov, V. A. 1974: 236
United Kingdom	Fabricius, J. C. 1781: 295
Nearctic	
Canada: Alberta	Pohl, G. R., Anweiler, G. G., Schmidt, B. C., Kondla, N. G. 2010: 60
Canada: British Columbia	Busck, A. 1904: 771
Canada: Manitoba, New Brunswick, Nova Scotia, Ontario	Pohl, G. R., Landry, J.-F., Schmidt, B. C., Lafontaine, J. D., Troubridge, J. T., Macaulay, A. D., van Nieukerken, E. J., DeWaard, J. R., Dombroskie, J. J., Klymko, J., Nazari, V. & Stead, K. 2018: 57
Canada: Quebec	Handfield, L. 1997: 30
Canada: Saskatchewan	Pohl, G. R., Landry, J.-F., Schmidt, B. C., Lafontaine, J. D., Troubridge, J. T., Macaulay, A. D., van Nieukerken, E. J., DeWaard, J. R., Dombroskie, J. J., Klymko, J., Nazari, V. & Stead, K. 2018: 57
United States	Chambers, V. T. 1872: 27
United States: Atlantic States	Dyar, H. G. [1903]: 559
United States: Florida	Heppner, J. B. 2007: 241
United States: Illinois	Godfrey, G. L., Cashatt, E. D. & Glenn, M. O. 1987: 14
United States: Kentucky	van Orden Covell, C. 1999: 23
United State: Maine	Brower, A. E. 1984: 46
United States: Massachusetts	Forbes, W. T. M. 1923: 175
United States: Michigan	Nielsen, M. C. 1998: 4
United States: New York	De Prins, W. & De Prins, J. 2005: 127
United States: Ohio, Pennsylvania	Forbes, W. T. M. 1923: 175
United States: South Carolina	De Prins, W. & De Prins, J. 2005: 127
United States: Texas	Frey, H. & Boll, J. 1876: 210
United States: Vermont	Grehan, J. R., Parker, B. L., Nielsen, G. R., Miller, D. H., Hedbor, J. D., Sabourin, M. & Griggs, M. S. 1995: 5
Oriental	
India	Fletcher, T. B. 1933: 62

Scouting of the genus-level characters in *Caloptilia stigmatella*

Under the latest classification of Gracillariidae (Kawahara *et al*. 2017), 25 genera including *Caloptilia* are grouped into the subfamily Gracillariinae Stainton, 1854. Having a robust phylogeny of family level taxa, there is now a need for re-evaluation of morphological characters of diagnostic importance at the genus-level, so the generic delineations could be completed. The critical re-examination of the diagnostic characters in the historical Fabrician type specimen became crucial for reconsidering the further re-verification of species assigned to *Caloptilia*.

The morphological diagnostic characters for *Caloptilia* were verified by Vári (1961) and Kumata (1982), who established 8 subgenera within *Caloptilia* on the basis of general morphological characters of the imago, including forewing venation, internal characters of genitalia, and especially the chaetotaxy of the last instar larvae. As both revisers (Vári 1961; Kumata 1982) mentioned in their generic diagnoses, many diagnostic characters can vary from species to species and it is up to revisers to determine the corresponding differences which fall within generic or species levels. Wing venation was traditionally used in Gracillariidae to separate closely related genera (Ely 1918). The position of veins and the degree of their coalescence may vary, however, in combination with other characters (ecology, external and internal morphology of adults, pupae and larvae). Wing venation, especially in the absence of an abdomen, is one of the key generic diagnostic characters in Gracillariidae, e.g., the subgenera in *Caloptilia* were defined by Kumata (1982) whether radial veins distal to the discoidal cell are well separated at their bases, and whether forewing veins M_2 and M_3 are connate or short stalked at their bases.

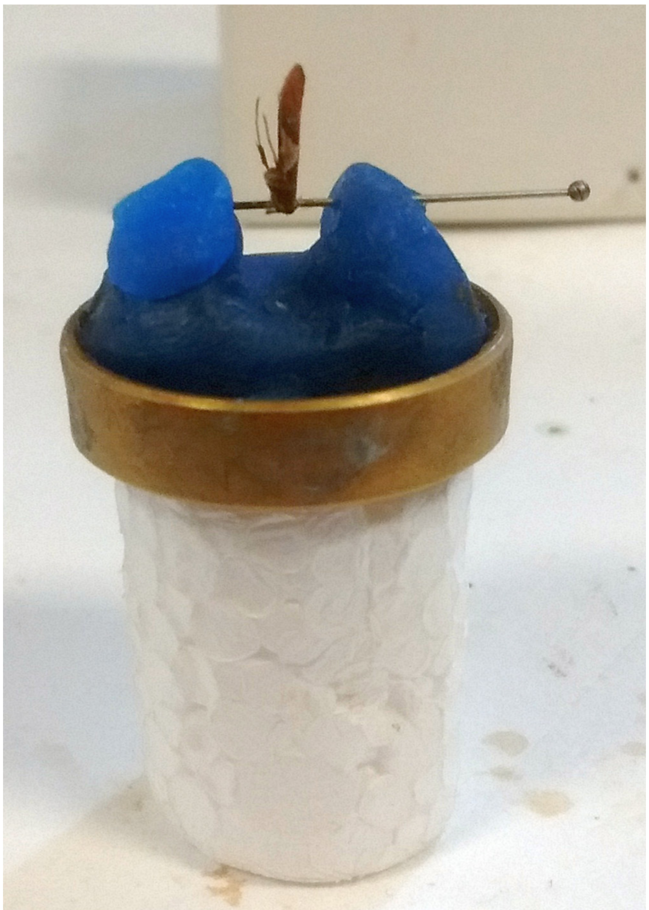

FIGURE 3. *Caloptilia stigmatella* type. Fabrician type *C. stigmatella* secured in position with dental wax ready for scanning.

The determination of vein position in the forewing and hindwing of the type species of *Caloptilia* represented by the type specimen *Caloptilia stigmatella* Fabricius, 1781, despite slight variation of some radial and median veins in the degree of coalescence, indicates the group of species which are surely associated with the generic name *Caloptilia.* Since Fabricius first described *C. stigmatella* over two centuries ago many moths with very similar wing patterns have been incorrectly assigned to this group. This group is in desperate need of new characters to help make taxonomic sense of the group at a generic and specific level (Arévalo-Maldonado in prep.).

Micro-CT scanning and virtual descaling of wings

The lack of genitalia prompted taxonomic researchers to use micro-CT to investigate potential taxonomic characters in the wings. To reveal the wing veins by conventional methods would typically involve detachment of the wing and removal of the scales, an undesirable option for such a valuable and important specimen.

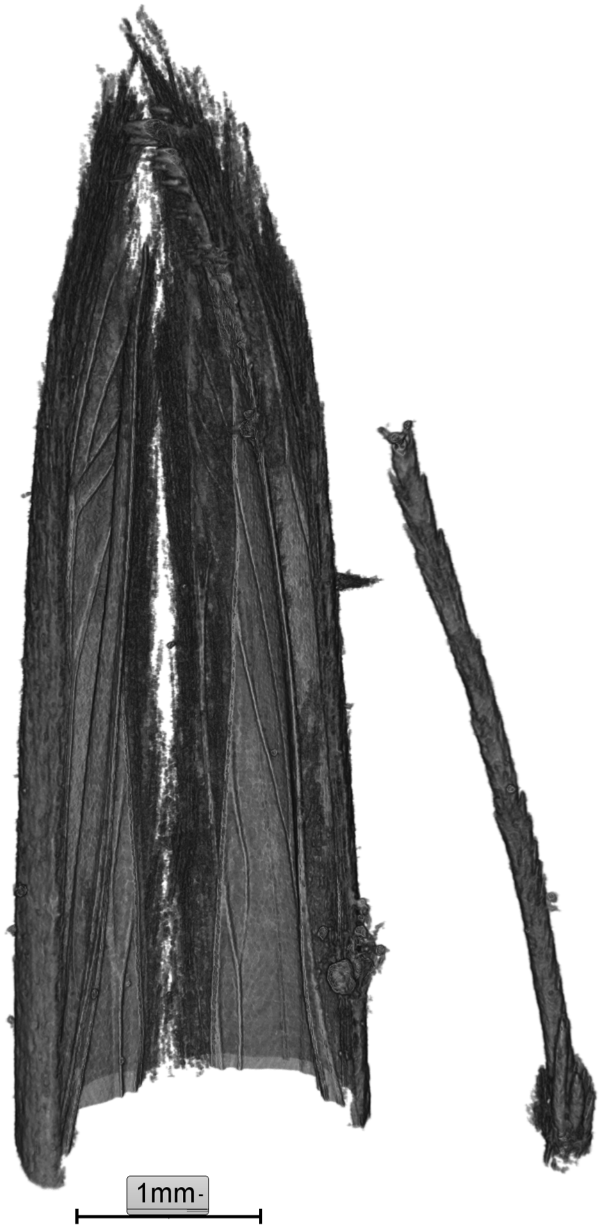

FIGURE 4. *Caloptilia stigmatella* type. Initial scan of *C. stigmatella* holotype wings targeted above the pin.

A test scan was performed on a classically set Victorian specimen on a headless micro-pin with its wings spread. The pin was inserted vertically onto a stage into dental wax. The pin served as the axis around which the insect was scanned. Artefacts from the pin almost entirely obscured the insect in the initial scans. Metal pins seriously affect the quality of a micro-CT scans because of their very high X-ray attenuation, causing artefacts in the reconstructed data (Simonsen & Kitching 2014). Next the type specimen was scanned; a different arrangement was required as the specimen has been mounted with closed wings on a longer pin. The pin was fixed horizontally using dental wax, with the head of the moth pointing down below the pin shaft and the wings above it (Fig. 3). This configuration allowed the closed wings to be scanned in their entirety whilst excluding the pin from the scan, thus avoiding attenuation (Fig. 4). Careful attention to the alignment and positioning of the specimen ensured that the specimen could be rotated safely within the scanning chamber without damage. The sample was scanned using a micro X-ray CT (Bruker Skyscan 1172) at 39kV and 100uAmps with a voxel size of 1.96um/pixel. Images from the micro X-ray CT were reconstructed into an image stack using nRecon (Ver. 1.6.9.18; Bruker). From the reconstructed image stack, an image stack for each wing was created with CT Analyser (Ver. 1.14.10.0+; Bruker) by using the region of interest (ROI) selection tools (Figs 5, 6). The polygon selection tool was used to surround the wing of interest across several layers of the reconstructed image stack; interpolation was used to select the rest of the wing based on the shape of the drawn polygon across the remaining image stack. Using the ROI, each wing was exported as a separate image stack for visualization and further processing in Amira (Ver. 6.3; Fisher Scientific / FEI).

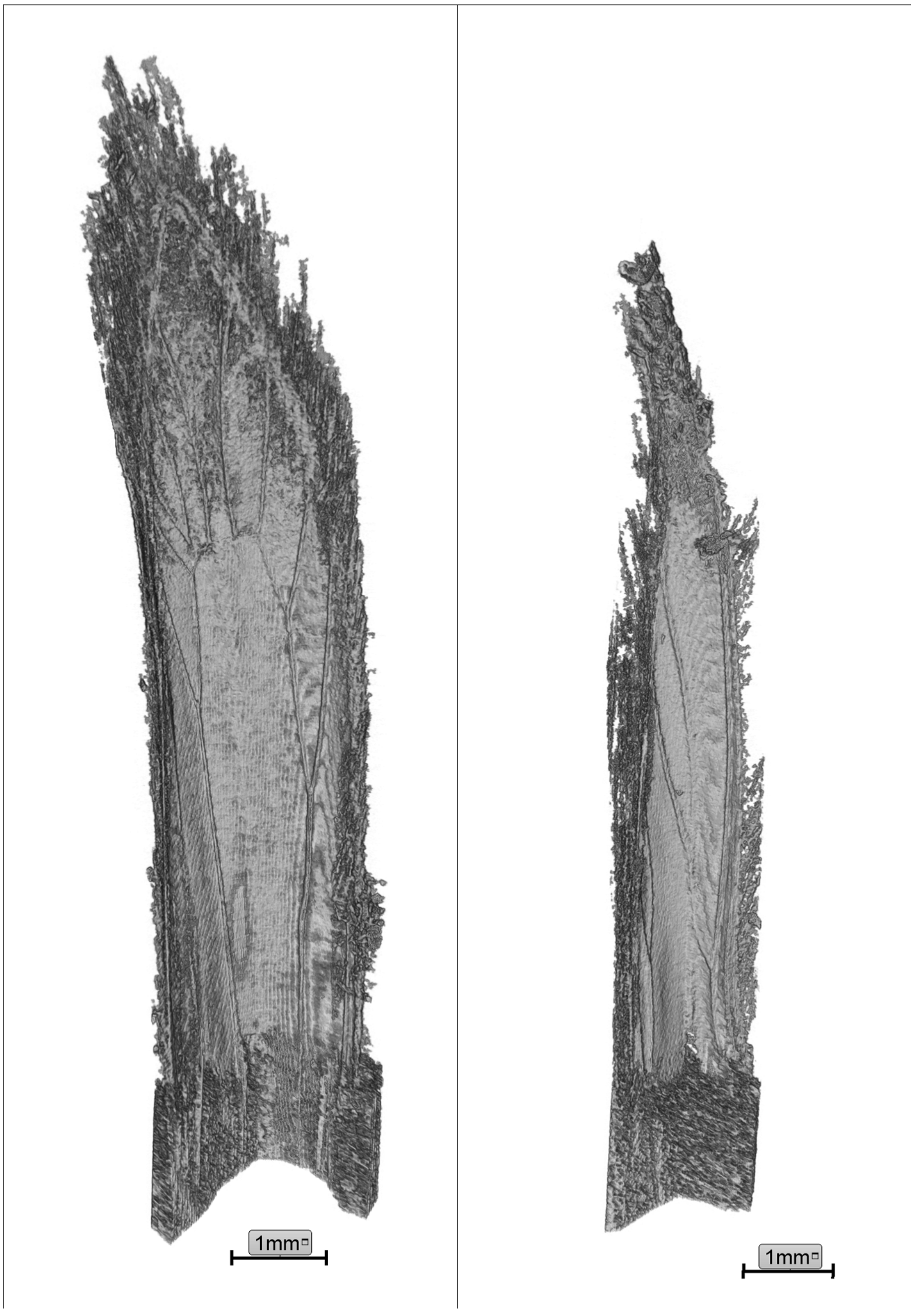

FIGURE 5. *Caloptilia stigmatella* type. 5. Visualisations of left fore and hindwings of *C. stigmatella* holotype.

Taxonomic data verification

Re-verification of characters obtained from the observation and virtual descaling of the Fabrician holotype of *Caloptilia stigmatella*

For the diagnostic morphological and ecological characters of the genus *Caloptilia* we follow Vári (1961), Kumata (1982) and De Prins (1985); for molecular diagnostic characters we follow Kawahara *et al*. (2017). The wing venation characters of the holotype of *C. stigmatella* are reconstructed following the procedure described above. The schematic visualization of veins obtained by micro-CT scanning is presented in Fig. 7 and the description is as follows:

Forewing narrow, lanceolate, bluntly pointed, apically with 13 veins (Fig. 7), discoidal cell slightly dilated distad with distal margin nearly vertical, apical part with 9 veins (R_2, R_3, R_4, R_5, M_1, M_2, M_3, CuA_1, CuA_2). all radial veins well separated at their bases, vein R_1 arises near base of forewing and ends on costal margin at about 3/5, R_2 arises from apical 1/3 of cell, R_3 arises from subapex of discoidal cell, R_4 arises from apical angle of discoidal cell, R_5 arises from the apical margin of the discoidal cell, M_2 and M_3 arise from tornal angle of discoidal cell. short stalked, vein CuP distinct only in apical part, vein An simple, curved, connected with dorsal margin of forewing near middle, vein R_1 obsolete at base.

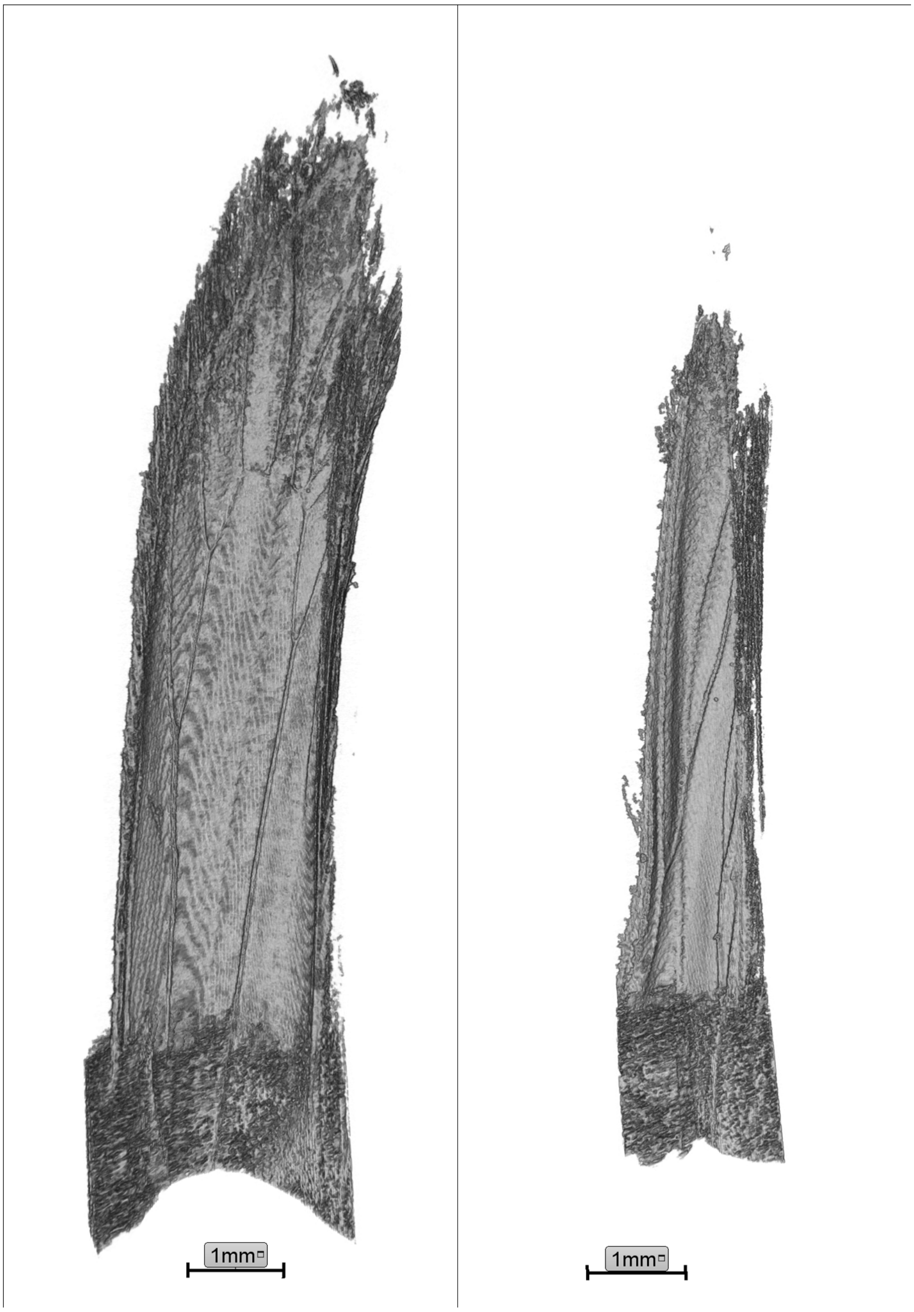

FIGURE 6. *Caloptilia stigmatella* type. Visualisations of right fore and hindwings of *C. stigmatella* holotype.

Hindwing 85% as long and 78% as wide as forewing, narrowly lanceolate, sharply pointed with 8 veins and with open cell between M_2 and M_3; vein R_{2+3} short and distinctly separated from Sc+R_1 and Rs; veins M_1 and M_2 long stalked, veins M_3, CuA_1 and CuA_2, placed at equal distance from each other at dorsal margin of hindwing, vein M_3 arises from near middle of vein CuA_1.

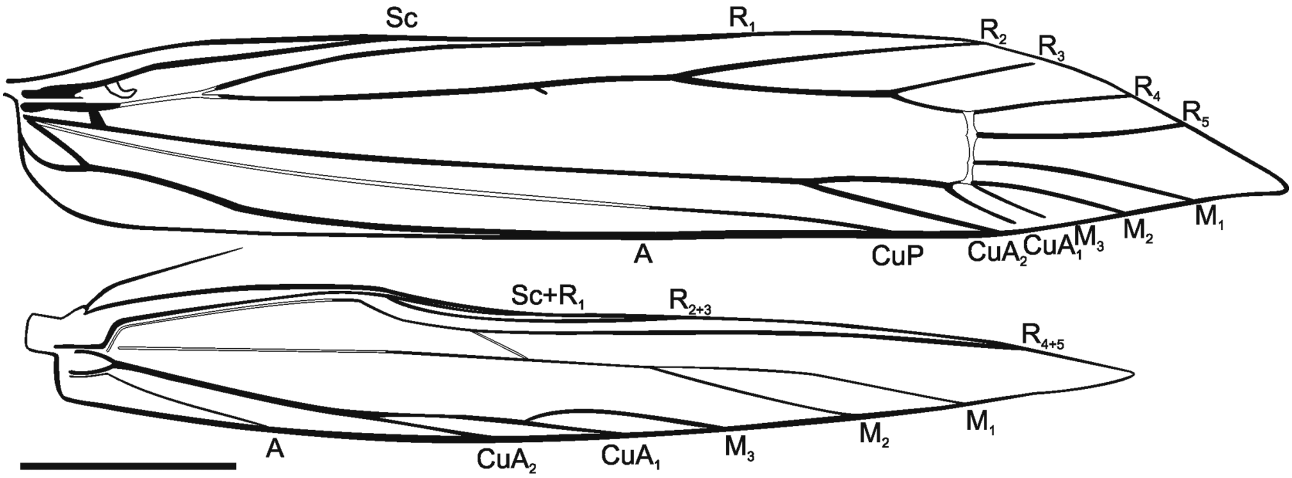

FIGURE 7. The schematic visualization of wing venation in the holotype of *C. stigmatella*.

Conclusion

Micro-CT was the only possible tool to study the wing venation of the type specimen of *Caloptilia stigmatella* Fabricius, 1781 in a non-destructive way. The scans of the fore- and hindwing were successful. Finally, after 237 years taxonomists will now be able to study and compare the matrix of veins in other congeneric species and hopefully delineate the genus *Caloptilia* and start to resolve some of the taxonomic ambiguity surrounding this leaf-mining family.

Acknowledgments

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