Micro-CT imaging in species description: exploring beyond sclerotized structures in lichen moths (Lepidoptera: Erebidae, Arctiinae, Lithosiini)

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Abstract

X-ray micro-computed tomography imaging (micro-CT) is valuable for systematic research since it permits the non-destructive scanning and imaging of internal structures of scarce species and/or type specimens. Additionally, micro-CT allows one to view the morphology and imagine the functional anatomy of structures in their natural anatomical position without deformations that typically occur using classical dissection protocols.

In this study, we describe two new species of lichen moths (Lepidoptera: Erebidae, Lithosiini) from the Atlantic Forest in eastern Brazil: *Nodozana heliae* Moraes **sp. nov.** from Rio de Janeiro state and *Epeiromulona pataxo* Moraes & Aguiar **sp. nov.** from Bahia state. The male and female genitalia and wing morphology were examined employing non-destructive micro-CT, subsequent 3D model reconstruction, 360-degree spinning animations, and 2D images from different angles. Those were compared against classical genitalia dissections from the same specimens. We conclude that techniques complement each other, micro-CT being particularly useful in studying wing venation, sclerotized internal structures and muscles. At the same time, classical dissection helps study membranous structures, particularly in the female genitalia, abdominal skin, and specialized scales on the male 8th sternite.

Keywords: integrative taxonomy, x-ray micro-computed tomography, morphology, muscles, DNA barcode.

Introduction

Lepidoptera, commonly called butterflies and moths (or buttermoths), are covered with scales. The scales form a wide variety of diagnostic colors and patterns, particularly on wings but also on other body parts. For that reason, they are extensively used in Lepidoptera systematics. The scales also hide many structural details, often relevant in systematics. These include wing venation, which is particularly important in suprageneric classification. Scales

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also hide modified sclerites, conventional setae, spikes, and secondary sexual characters, including the eversible scent-producing coremata (Scoble 1995, Kristensen 1999, Kristensen 2003). Other internal structures, which are important in systematics, include the reproductive organs and sclerites of the thorax and metathorax, to mention a few. These hidden structures have traditionally been studied using various dissection techniques, the most requiring at least partial destruction (e.g., Hardwick 1950, Sihvonen 2001, Moraes & Duarte 2009, Murillo-Ramos *et al*. 2016). Also, learning these dissection techniques requires extensive training. Only some experts can master this "craftsmanship" in such detail that museums allow the dissection of their valuable type or rare specimens.

As regards the genitalia, the dissection techniques such as potassium hydroxide (KOH) treatment (Robinson 1976), if carefully applied and structures cleaned, reveal the cuticle sclerotized or not very well, and those can be studied and imaged to the finest detail. The genitalia also contain membranous structures, which are more challenging to explore because they are transparent, delicate, and easily break or are detached from other structures during the dissection. This is the case, particularly in the female genitalia. A notable problem with the KOH treatment is that it readily dissolves the male-deposited spermatophores and female eggs inside the ovaries, or makes the muscles invisible, which means that important taxonomic information is lost. Staining enhances the study of membranous structures. Still, the problem remains that classical dissection methods damage some structures, and certain structures are even removed routinely to make other structures visible. Finally, dissected structures are routinely embedded in microscopic slides and mounted in an artificial position, such as the male valvae spread out. In some moth groups, the male genitalia stay unrolled (Pitkin 1986), and the vesica rests inside the aedeagus. Some of these practices are useful for taxonomic research and for storing extensive materials in museums. Still, it does not allow the

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subsequent study of the structures from various angles, which are helpful in taxonomy and functional anatomy research.

X-ray micro-computed tomography imaging (micro-CT) is a fast and non-destructive data acquisition technique that can complement traditional, partly destructive dissection methods in morphological studies. Since its first application in entomology 20 years ago (Hörnschemeyer *et al.* 2002), micro-CT is still relatively little used in insect morphology, mainly because the equipment is expensive and relatively few museums have it. The inspiring examples of micro-CT in Lepidoptera taxonomy include, among others, virtual dissections of reproductive organs (Simonsen & Kitching 2014), wing venation study on over 200-hundred-year-old type specimen (Robinson *et al*. 2018), and functional morphology of internal structures (Nath & Kunte 2020). Other benefits of the approach include the study of morphologies in their natural position without deformations, versatile post-processing of data (Garcia *et al*. 2017a), and the scrutiny of valuable material such as type specimens or scarce species without damaging the samples (Stoev *et al*. 2013, Garcia *et al*. 2017b).

Lithosiini, or Lichen moths, is a species-rich insect lineage whose subtribes and genus taxonomy are notorious, needing a modern integrative approach. The tribe includes approximately 3150 species, classified into 457 genera (Scott *et al*. 2014). Of these, 345 genera are classified as *incertae sedis* (Bendib & Minet 1999), and new species are described constantly (e.g. Durante & Apinda-Legnouo 2022, Volynkin *et al*. 2022a, b). The tribe is exceptionally species-rich in the Neotropical region (Scott *et al*. 2018), and in Brazil, there are 212 species classified in 52 genera (Moraes & Casagrande 2019). However, these numbers are underestimated given the scarcity of specialists working with Neotropical fauna and the potential existence of new records and species.

While the morphology of Lithosiini moths has been studied extensively using classical methods, these moths have not been the target of micro-CT imaging earlier. We chose two

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undescribed lichen moth species as our study species. These originate from the Brazilian Atlantic Forest, one of the Earth's Biodiversity hotspots with high levels of diversity and endemism. The biodiversity and biomass in this area have been reported to be eroding at an alarming rate (de Lima *et al.*, 2020). We aim to explore how micro-CT imaging can enhance the study of morphological structures compared to classical dissections and how it can be applied in systematic research. Our study scrutinizes scale-hidden cuticle, wing venation or abdominal structures, and retracted and concealed genitalia *in situ*, suggesting the best approach to unveil the fine morphology of the parts.

Material and Methods

Abbreviations

MZUSP	Museu de Zoologia da Universidade de São Paulo, Brazil
ZMH	Finnish Museum of Natural History, University of Helsinki, Finland
ZUEC	Zoological collection, Museu de Diversidade Biológica, Universidade Estadual de Campinas, Brazil

Sampling and identification

Moth collection was carried out in the Brazilian states of Rio de Janeiro and Bahia between 2016–2021 during the new-moon phase to enhance light attractiveness using a 500 W mixed light bulb and a white 2 m x 2 m sheet to attract the moths. Specimens were individually kept alive in small glass containers and then were killed with ethyl acetate just before the wing spreading.

For identification, specimens were compared against relevant literature and online sources (Seitz 1914, 1943, taxonomy section on Barcode of Life Data Systems https://v4.boldsystems.org/), to material in relevant collections (ZUEC, MZUSP, ZMH), including both type and non-type specimens, and DNA barcodes (658 bp region near the 5'

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terminus of the COI mitochondrial gene) were compared against the genetic material available on BOLD (Ratnasingham and Hebert 2007, 2013) and GenBank (Benson *et al*. 2013). Genetic divergences between sequences were calculated using the number of base differences between sequences. Voucher specimens are deposited at the institutions mentioned above (details are given under the *Species description* chapter).

DNA extraction and PCR protocol

Three legs were removed from each specimen shortly after collection and before the wing spreading. Sampled legs were preserved dry and stored in 1.5 ml tubes at - 20˚ C. Total genomic DNA was extracted with QIAcube DNA extraction robot (Qiagen, Netherlands) using DNeasy Blood & Tissue Kit standard protocol with final elution in 100 µl elution buffer. The 5' end (barcode region) of the mitochondrial gene cytochrome oxidase subunit I (COI, 650 bp) was amplified for the SSM3 sample with LepF1 (Wilson, 2012) and HCO (Folmer *et al.* 1994) primers. The barcode for the SSM4 sample was amplified in two parts: the first half (~330bp) with LepF1 and mLepR1 (Wilson, 2012) primers and the end half (~450bp) with Beet (Simon *et al.* 1994) and HCO (Folmer *et al.* 1994) primers.

Polymerase chain reactions (PCRs) were performed with 13 µl total volume containing 3 µl of extracted DNA, 2 µl of H_2O milli-Q, 6.5 µl of 2x MyTaq HS red mix (Bioline Co., UK), and 0.75 µl of each primer (10 mM). PCR products were amplified as follows: 96˚C for 7 minutes, followed by 40 cycles of 96˚C for 30 seconds, 50˚C for 30 seconds, and 72˚C for 90 seconds, and a final extension period of 72˚C for 10 minutes. Amplicons were purified by mixing 5 µl of PCR product with 2 µl of 1/10 H_2O milli-Q diluted ExoSAP-IT (Thermofisher Scientific, USA). Purification was run in a PCR machine: 37˚C for 15 min and 80˚C for 15 min. Purified products were sent for Sanger sequencing to FIMM (Institute for Molecular Medicine Finland).

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295 *Morphological examination*

296 We first imaged the adult specimens using a non-destructive micro-CT approach and
297 tried to identify external and internal morphological structures *in situ*. Following this, both the
298 male and female abdomen and genitalia of both new species were dissected. When data from
299 both approaches were available, this allowed us to refine homology interpretations in both
300 approaches.

301 **Micro-CT imaging**. Each adult specimen was pinned using a minute pin, attached to a
302 foam cube, traversed by a standard insect pin. To avoid noise and artifacts resulting from the
303 standard insect pin holding the foam cube, the pin was pushed down to exclude it from the
304 scanning area. The samples were imaged using Nikon XT H 225 micro-computed
305 tomography. Scans were performed using a multi-metal target with a molybdenum setting,
306 with 73-74 kV beam energy, 94-95 uA beam current, 500 ms exposure time, and 4476
307 projections with four frames per projection. Detector binning was set to 1x1 and gain to 24
308 dB. Imaging was conducted using limited dynamic range after performing comparisons that
309 showed no visible differences between the longer full dynamic range scans and the faster
310 limited dynamic range scans. Adequate pixel size ranged from 3.27 to 7.27 µm between the
311 scans. Projections were processed using Nikon CT pro-3D, and the 3D models were exported
312 to VGSTUDIO 3.5.2 (Volume Graphics GmbH, Heidelberg, Germany) in 16-bit. The 3D
313 models were visualized using two renderer modes, volume renderer (Phong) and X-ray, and
314 they were pseudo-colored to visualize density. Virtual section stacks in the three principal
315 planes (coronal, sagittal, and axial) were exported in JPG format.

316 The external morphology and color pattern were analyzed following usual protocols
317 (Winter 2000).

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Dissection. The abdomens and genitalia of females and males were dissected following standard methods (Hardwick, 1950). The male aedeagus is shown with retracted vesica to allow comparison with older literature and everted vesica. The vesica was everted via the caecum cut open by placing the aedeagus inside a hypodermic syringe (Sihvonen, 2001). Some structures were photographed during dissection *in situ* to allow an optimal angle for observing and illustrating specific structures. Numerous dissected structures shown in the plates were shot in two to six images at different depths of focus, using a Leica DM1000 microscope and Leica DFC295 camera, and combined into single images using image-stacking software in Adobe Photoshop CC v.20.0. For interpretation and descriptions of the genitalia structures we followed the procedures outlined in Moraes & Duarte (2015), and terminology of the male genitalia follows (Pierce 1909, Sibatani *et al.* 1954, Okagaki *et al.* 1955, Klots 1956, Ogata *et al.* 1957, Birket-Smith 1974). Female genitalia follows (Pierce 1914, Klots 1956, Mutuura 1972, Galicia *et al.* 2008). The muscle nomenclature follows Kuznetzov *et al*. (2004).

A total of 19 specimens belonging to the two new species were examined. Details are given under each species below.

The electronic version of this article in Portable Document Format (PDF) will comply with the International Commission on Zoological Nomenclature (ICZN) rules. Hence the new names in the electronic version are effectively published under that Code from the electronic edition alone. This published work and its nomenclatural acts have been registered in ZooBank, the online registration system for the ICZN (urn:lsid:zoobank.org:pub:68906FAC-208D-48D7-B69C-4ABDE6CFA0D6, urn:lsid:zoobank.org:act:F97C4D7C-65A3-4EEC-8D34-F84D5C7346EE, urn:lsid:zoobank.org:act:D4637E11-169E-45D0-8A25-6A61F68939A5). The ZooBank LSIDs (Life Science Identifiers) can be resolved, and the associated information viewed

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Results

Micro-CT imaging

The micro-CT scanning and post-processing of 3D models allowed us to visualize clearly and in a non-destructive manner the wing venation and wing folds in both sexes (2D image in Fig. 1, 3D spinning video on Supplementary material 1), several sclerotized structures in the male abdomen, and several sclerotized structures and muscles in the male genitalia (Fig. 6). The visible male structures include, for instance, the posterior margin of the 8th abdominal tergite, tegumen, uncus, valva, transtilla, aedeagus and cornuti (2D image in Fig. 6, 3D spinning video on Supplementary material 2).

Genital muscles were visible in the 3D models (Fig. 6, Supplementary material 2). Those include

- the m1 muscle. The depressor of the uncus is a broad longitudinal muscle extending ventromedially from the anterior margin of the tegumen to the base of the uncus;
- the m5 (7) muscle. The flexor of the clasper is an intravalval muscle extending from the anterior part of the valva, in the region of the sacculus, to the central part of the valva. These muscles bend the valvae transversally, clasping the female abdomen during the copulation;
- the m6 (5) muscle. The protractors of the aedeagus originate on the dorsolateral region of the vinculum and insert on the base of the aedeagus;
- the m7 (6) muscle. The retractors of the aedeagus extend from the saccus and insert mid-ventrally on the aedeagus.

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As regards the membranous structures embedded in the abdomen, micro-CT scanning and post-processing of 3D did not produce clear images, and homology interpretation was difficult. This was mainly because the density of membranous structures and body fat were similar. As regards the female genitalia, which are primarily membranous, most of the structures could be identified only via the sequential study of the sagittal slices (Supplementary material 3, 4). In the sagittal section, we were able to detect the sclerotized signa, antrum, and ductus bursae, which we used as reliable morphological anchor points, and subsequently, from these images, it was possible to infer the outer surface of the membranous corpus bursae and ductus bursae (Fig. 7). By carefully adjusting the 3D histogram it was possible to identify other regions with very low density, representing the interior of the corpus bursae, membranous ducts, and margins of the pheromone glands (Fig 7).

Micro-CT imaging cannot be used to study the shape of eversible membranous structures. Among these is the male vesica, which we explored using the method of Sihvonen (2001), demonstrating their complexity in both species.

Species description

Comparison of our material, using both morphological and DNA barcode data, against described species of Lithosiini did not result in a positive match, thus suggesting our specimens belong to undescribed species. We provide the formal descriptions below.

Nodozana heliae Moraes **sp.nov.** (Figs. 1-3, 7)

Diagnosis (♂ and ♀). Prothoracic collar orange. Dorsal surface of the forewing with several subrectangular white maculae, orange maculae on the wing base seahorse-shaped, orange maculae on the submarginal region hammer-shaped with an elliptical black spot inside. Compared with other *Nodozana* species, the wing pattern with white squares is idiosyncratic. Only *Nodozana toulgoeti* Gibeaux, 1983 has a similar wing pattern but without the red basal

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macula on the forewing. We combine taxon *heliae* to *Nodozana*, based on DNA barcode data (see below) and similar wing pattern with *N. toulgoeti*, but highlight those genus-level systematics of Neotropical lichen moths are poorly resolved, and more research is needed. *N. toulgoeti* is known for French Guiana.

Description (♂ and ♀). Head. Brown. Frons brown, vertex yellow. Labial palp brown. **Thorax**. Predominantly brown. Prothoracic collar orange; prothoracic coxa brown. Tegulae beige. **Wings**: Venation as in Figure 1b: Wingspan 15,5–17,5 mm (n=4). Forewing background dark brown; basal orange seahorse-shaped macula; white subrectangular maculae distributed on post-basal, discal, post-discal and marginal regions; a post-discal region with orange hammer-shaped maculae between M_1 and CuP veins; elliptical black macula between M_1 and M_3; ventral surface of forewing dark brown, with narrow orange stripe near wing apex; maculae obscured by dark brown scales. Hindwing yellow, apex with a dark brown macula, outer margin outlined by dark brown scales; ventral surface similar. **Abdomen**. Dorsally light brown, posterior margin of segments A_2-A_8 outlined by dark brow scales; ventrally similar, 7th sternite margins weakly sclerotized, posterior margin concave. 8th sternite with anterior and lateral margins sclerotized, setose coremata medially. **Male Genitalia**: Tegumen trapezoidal in dorsal view, anterior margin concave. Uncus hooked; apex acute. Valva entire, sub-rectangular; sacculus developed, fold on the inner surface, oriented towards the distal-medial axis. Transtilla sclerotized, inverted U shaped. Juxta membranous. Subscaphium sclerotized. Aedeagus rectilinear, sclerotized, with micro spicules on the anterior portion near vesica; caecum rounded, foramen lateral; vesica large, with three large diverticula, single spiniform cornutus. **Female Genitalia**. Seventh sternite smooth. Ostium membranous. Antrum sclerotized, with microspicules. Ductus bursae are short and membranous. Corpus bursae extending beyond the seventh sternite, signa as two patches of

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spines on the lateral portion of the bursa. Lamella antevaginalis and postvaginalis absent. Papillae anales narrow, setose.

Etymology. The specific epithet is granted in honor of Maria Hélia de Souza Moraes, mother of the first author. It is also a reference between the golden scales on the forewing and Helios, the sun's personification in Greek mythology.

Distribution. The only record for this species is from Itatiaia, a montane-dense ombrophilous forest of medium and high altitudes in Rio de Janeiro State, Brazil.

DNA barcode data. (n=1) from Brazil: Rio de Janeiro. Based on nucleotide blast function on GenBank, the nearest lichen moth species is *Nodozana toulgoeti* from French Guiana, with a 7% difference.

Type series. HOLOTYPE ♀: BRAZIL: **Rio de Janeiro**: Itatiaia, Parque nacional do Itatiaia, Alojamento 12, 01-04/viii/2016, Simeão Moraes, Tamara Aguiar, André Taciolli leg., label: LEPSM 551 (ZUEC). PARATYPES BRAZIL: 1♀ **Rio de Janeiro**: Itatiaia, Parque nacional do Itatiaia, Alojamento 12, 01-04/viii/2016, Simeão Moraes, Tamara Aguiar, André Taciolli leg., labels: Simeão Moraes Genitalia 516, LEPSM 1093 (ZUEC); 1♂ **Rio de Janeiro**: Itatiaia, Parque nacional do Itatiaia, Casa do Pesquisador 12, 05-12/iv/2021, 22°27'19.9'' S 44°36'29'' W, Simeão Moraes leg., labels: Pasi Sihvonen Prep. Number 2870, LEPSM 1382 (ZUEC); 1♀ **Rio de Janeiro**: Itatiaia, Parque nacional do Itatiaia, Casa pesquisador, 05-12/iv/2021, 22°27'19.9''S 44.36'20.0''W, Simeão Moraes leg., labels: LEPSM 1439, SSM DNA sample 3, specimen ID http://id.luomus.fi/GBT.11 (ZMH).

Epeiromulona pataxo Moraes & Aguiar **sp.nov.** (Figs. 1, 4, 5)

Diagnosis (♂ and ♀). The forewings are dorsally white, with several small black maculae on the proximal portion and outer margin with reddish scales. Hindwing uniform salmon colored. The forewing pattern with white/beige background and black maculae/dots are shared with

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other species in *Epeiromulona*, but the reddish outer margin is idiosyncratic in *Epeiromulona pataxo*.

Description (♂ and ♀). Head. White. Frons brown, vertex white. Labial palp white. **Thorax**. Predominantly white. Prothoracic collar white; prothoracic coxa white. Tegulae white. **Wings**: Venation as in Figure 1a. Wingspan 12,75–13,3mmmm (n=15). Forewing background white with two proximal maculae: elliptical on costal margin, rounded on the trunk of A vein; four maculae on the medial region: two elliptical on costal margin, subrectangular at the discal cell, subrectangular at A vein; a submarginal region with two sinuous stripes, proximal longer than distal; outer margin with reddish scales; ventral surface reddish with black maculae fused along the basal length of costal margin, black stripes join on the subapical region. Hindwing dorsally salmon-colored; ventrally salmon with apical black macula on the region of Rs and M1. **Abdomen**. Dorsally salmon-colored on A_2-A_4, reddish on A_3-A_8; ventrally salmon. Male segments 7-8 are not differentiated. **Male Genitalia**: Tegumen subrectangular in dorsal view, anterior margin concave. Uncus hooked, with acute apex. Vinculum narrow. Valva is relatively immobile, setose, sub-triangular, apex rounded; sacculus developed, consisting of the fold on the inner surface, oriented towards the distal-medial axis. The saccus is large and subtriangular. Juxta sclerotized, subtriangular. Subscaphium smooth. Aedeagus rectilinear, sclerotized, two rows of spines on apex; ejaculatory bulb rounded, foramen lateral; vesica bilobated, cornuti on the larger lobe: micro spines medially and needle-shaped spines on distal part. **Female Genitalia**. **7th** sternite smooth. Ostium membranous. Antrum slightly sclerotized, smooth. The Ductus bursae is short and membranous. Corpus bursae is massive, extending beyond the seventh sternite, with two patches of signa, spines on the posterior portion, and weakly fused micro spicules on the anterior part. Lamella antevaginalis and postvaginalis not sclerotized. Papillae anales large, setose.

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Etymology. The specific epithet honors the Pataxós, indigenous people inhabiting the state of Bahia, Brazil, which is the location where the specimens were collected. A masculine name in apposition.

Distribution. The only record for this species is the National Park of Monte Pascoal in Porto Seguro, State of Bahia, Brazil. The region represents one of the last remnants of the Atlantic Forest, where the predominant vegetation is tropical rainforest. The physiognomic and structural aspect is like the dense and exuberant vegetation of the Hylian **Amazon**.

DNA barcode data. (n=1) from Brazil: Bahia. Based on the nucleotide blast function on GenBank, the nearest species is *Epeiromulona* sp. from Costa Rica, with a 7% difference.

Type series. HOLOTYPE ♂: BRAZIL: **Bahia**: Porto Seguro, Parque Nacional Monte Pascoal, Sede, 150m, 12-23/v/2021, 16˚53’16.13’’ S 39˚24’57.46’’W, Simeão Moraes leg., (ZUEC). PARATYPES BRAZIL: 1♀ **Bahia**: Porto Seguro, Parque Nacional Monte Pascoal, Sede, 150m, 12-23/v/2021, 16˚53’16.13’’ S 39˚24’57.46’’W, Simeão Moraes leg., (ZUEC); 7♂♂ **Bahia**: Porto Seguro, Parque Nacional Monte Pascoal, Sede, 150m, 12-23/v/2021, 16˚53’16.13’’ S 39˚24’57.46’’W, Simeão Moraes leg., (ZUEC); 2 ♂♂ **Bahia**: Porto Seguro, Parque Nacional Monte Pascoal, Sede, 150m, 12-23/v/2021, 16˚53’16.13’’ S 39˚24’57.46’’W, Simeão Moraes leg., (MZUSP); 2♂♂ **Bahia**: Porto Seguro, Parque Nacional Monte Pascoal, Sede, 150m, 12-23/v/2021, 16˚53’16.13’’ S 39˚24’57.46’’W, Simeão Moraes leg.. 1♂ **Bahia**: Porto Seguro, Parque Nacional Monte Pascoal, Sede, 150m, 12-23/v/2021, 16˚53’16.13’’ S 39˚24’57.46’’W, Simeão Moraes leg., Genitalia SSM A, specimen ID http://id.luomus.fi/GBT.12 (ZMH); 1♂ **Bahia**: Porto Seguro, Parque Nacional Monte Pascoal, Sede, 150m, 12-23/v/2021, 16˚53’16.13’’ S 39˚24’57.46’’W, Simeão Moraes leg., Pasi Sihvonen, prep. number 2868; Pasi Sihvonen, DNA sample 1544, specimen ID http://id.luomus.fi/GBT.13 (ZMH).

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Discussion

The two species described herein have idiosyncratic wing patterns, distinct from other lichen moth species in the Atlantic Forest biome. Although the geographical distribution recorded is narrow, we expect that these species might be found in other areas in similar habitats near Rio de Janeiro and Porto Seguro. These species may have been overlooked because of their small size, and secondly, the specialists investigating the Neotropical fauna of lichen moths are scarce.

Sclerotized and membranous structures

In Lepidoptera taxonomy, wing venation characters, in addition to abdomen and genitalia characters, are among the most diagnostic, routinely studied, and illustrated in publications (e.g., Carter & Kristensen 1998, Winter 2000, Moraes & Duarte, 2009). However, because Lepidoptera are covered with scales, these characters cannot be studied without scale removal. The widely used protocols are partly destructive, such as wing bleaching that removes color from scales (e.g., Moraes & Duarte 2009, Murillo-Ramos *et al.* 2016), or KOH approach that dissolves fat body but makes the sclerotized structures in the abdomen and genitalia visible (e.g., Hardwick, 1950, Robinson 1976). Although membranous structures may be visible to some degree after KOH treatment, their visualization also depends on the successful application of stains such as Chlorazol Black and Eosin. In these approaches, some membranous structures, such as ducts, are routinely removed, and other abdominal structures, such as androconial scales and pheromone glands, are rarely illustrated.

We noted that non-destructive micro-CT imaging and post-processing of 3D models provided relatively easy and informative access to specific sclerotized and non-sclerotized structures. The 3D wing models made it possible to identify veins in a detailed manner and folds on the wing membrane close to the anal veins in both wings. Sometimes these folds are

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mistakenly identified as veins on 2D images. As regards the male genitalia, 3D models clearly illustrated the majority of the sclerotized structures, such as tegumen, uncus, transtilla, aedeagus, and valvae (Fig. 6). Non-sclerotized structures were difficult to visualize, including membranous juxta in the male genitalia of *Nodozana heliae* Moraes sp. nov. and membranous structures in the female genitalia.

Muscles

Four non-sclerotized genital muscles were visible in the 3D models (Fig. 6, Supplementary material 2): m1, m5 (7), m6 (5), and m7 (6). Because micro-CT models illustrate the structures in their natural position, it allowed for inferring the precise origin and inserting regions of muscle fibers and their naming. For example, the anatomical position of the aedeagus on the genital capsule allowed the distinction between the protractor and retractor muscles of the aedeagus (m6 (5) and m7 (6)) (Figure 6). The former appears longer, with origin in the dorsomedial portion of the vinculum and insertion in the base of the aedeagus, close to the caecum; the latter set of muscles are shorter and originates in the most ventral portion of the vinculum (saccus) and inserts in the central-ventral portion of the aedeagus. We did not detect the presence of muscle m8 (3), which originates in the median part of the vinculum and inserts at the distal margin of the juxta, being an indirect abductor of the valvae (Kuznetzov *et al*. 2004). The absence of m8 (3) might be correlated with the lack of a sclerotized juxta in the *Nodozana heliae* Moraes sp. nov.

The abductor and adductor musculature of the valva m3 (2) and m4 were not clearly distinguished in the 3D models. Although it is possible to identify something similar to muscles in the region between the transtilla and the valva, a better rendering of the 3D models is necessary to accurately assess the presence of these muscles, as well as the retractor muscle of the vesica (m21), usually located inside the base of aedeagus (Kuznetzov *et al*. 2004). In

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our study species, the valvae are morphologically simple, without all morphological subdivisions proposed by Sibatani *et al.* (1954), but we identified the intravalval muscle m5 (7), the flexor of clasper. This indicates that it may be possible to locate more muscles in species with more complex valva, such as some lichen moths species in the genus *Inopsis* Felder, 1874, in which it is possible to identify all six subdivisions of valva. Further, the micro-CT approach may help to access the configuration of the intravalval musculature and to investigate whether the valva subunits have intrinsic musculature. This may provide new evidence on serial homology studies for the appendicular origin of the male genitalia and allow the evaluation of characters' plesiomorphic or apomorphic state in a phylogenetic context (Moraes 2014).

Classical approaches are superior for specific structures

The classical, partly destructive dissection methods allowed us to identify some membranous structures in better detail (Figs. 2-5). These include, for instance, the abdominal skin and specialized scales on the male 8th sternite (in *Nodozana heliae* Moraes sp. nov., the ductus ejaculatorius in males, and the ductus bursae, corpus bursae and papillae anales in females. Details of the male vesica, i.e., shape, size, and position of sclerotized structures and membranous diverticula, were only visible in the classical approach (Sihvonen 2001) (Figs 2, 4). In lichen moths, the vesica is somewhat complex and very informative for taxonomy, with some species showing several diverticula and different shapes of cornuti (Durante & Apinda-Legnouo 2022, Volynkin *et al*. 2022a, b). Our results support the earlier view: also, in the smallest lichen moths, the vesica is complex and contains diagnostic characters.

Conclusion

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Our results demonstrate that micro-CT scanning combined with traditional dissection protocols can create virtual dissections of the male genitalia in lichen moths, where most diagnostic structures are visible. Furthermore, 3D reconstructions have the advantage of visualizing the morphological structures, such as the genitalia muscles and wing venation, without scale removal. Muscle information is usually lost with KOH, and wing colors are lost if bleaching is used.

Although the 3D reconstructions presented here are promising, we emphasize that micro-CT scanning cannot fully replace the abdomen and genitalia dissections in Lepidoptera for systematics and taxonomy purposes. Many specimens will not produce satisfactory 3D models, and membranous structures embedded inside soft tissue, such as body fat, appear problematic. This is often the case for the female genitalia in Lepidoptera, where the genitalia are almost entirely membranous, as for some critical traits in the male genitalia, such as the vesica. The latter's shape and number of lobes can only be fully understood when the structure is physically everted and shown as maximally inflated. Wings are somewhat 2D structures, and for the study of wing venation, we recommend micro-CT scanning as the first approach. We also acknowledge that further post-image processing of raw data could allow identifying additional structures which were not visible to us.

The advantages of using micro-CT in systematics are undeniable. First, it represents a non-destructive method that can study the type specimens and/or rare species. Second, it has the potential to access information on the morphology and the functional anatomy of structures in their natural anatomical position, which otherwise would be deformed or lost with classical dissection protocols. The use of micro-CT offers new opportunities for enhancing taxonomic descriptions and comparative studies, e.g. via video files, broadening the utility of morphological characters also in other disciplines in biology.

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Page 3: [5] Commented [F16] **Don't panic!** **06/01/2023 10:30:00**

Membranes are still made by cuticle. Please detail difficulties in studying membranes.

Page 3: [6] Commented [F17] **Don't panic!** **06/01/2023 11:37:00**

Please solve the passive form. And clarify the sense of “detached”, please.

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Please detail the “important taxonomical information”

Page 3: [9] Commented [F22] **Don't panic!** **06/01/2023 10:37:00**

Well, KOH or NaOH, are purposely used to dissolve tissues that take different shapes and change in time also depending on fixation techniques to reveal the stable cuticular features. Carachter stability in time and space is a crucial requisite to choose a taxonomical character.

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Please propose a reference for the routine removal for visualise structure. Thanks.

Page 3: [12] Commented [F24] **Don't panic!** **06/01/2023 10:43:00**

Structure damages, I presume your use “structure” in a very broad sense, may occur but more frequently the slide mounting technique include clearing/diaphanising steps - i.e. using Essig’s Aphid fluid - allowing to observe the cuticle via the piece, thus avoiding the need to dissect.

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