DNA mini-barcodes in taxonomic assignment: a morphologically unique new homoneurous moth clade from the Indian Himalayas described in *Micropterix* (Lepidoptera, Micropterigidae)

David C. Lees, Rodolphe Rougerie, Christof Zeller-Lukashort & Niels P. Kristensen

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The first micropterigid moths recorded from the Himalayas, *Micropterix cornuella* sp. n. and *Micropterix longicornuella* sp. n. (collected, respectively, in 1935 in the Arunachel Pradesh Province and in 1874 in Darjeeling, both Northeastern India) constitute a new clade, which is unique within the family because of striking specializations of the female postabdomen: tergum VIII ventral plate forming a continuous sclerotized ring, segment IX bearing a pair of strongly sclerotized lateroventral plates, each with a prominent horn-like posterior process. Fore wing vein R unforked, all Rs veins preapical; hind wing devoid of a discrete vein R. The combination of the two first-mentioned vein characters suggests close affinity to the large Palearctic genus *Micropterix* (to some species of which the members of the new clade bear strong superficial resemblance). Whilst absence of the hind wing R is unknown in that genus, this specialization is not incompatible with the new clade being subordinate within it. A 136-bp fragment of Cytochrome oxidase I successfully amplified from both of the 75-year-old specimens strongly supports this generic assignment. Translated to amino acids, this DNA fragment is highly diagnostic of this genus, being identical to that of most (16 of the 26) *Micropterix* species studied comparatively here, 1–4 codons different from nine other species (including *Micropterix wockei* that in phylogenetic analyses we infer to be sister to other examined species), whilst 7–15 codons different to other amphiplegenopteran genera examined here. A dating analysis also suggests that the large clade excluding *M. wockei* to which *M. cornuella* belongs appeared <31 million years ago. These findings encourage discovery of a significant radiation of *Micropterix* in the Himalayan region. Our analysis has more general implications for testing the assignment of DNA mini-barcodes to a taxon, in cases such as museum specimens where the full DNA barcode cannot be recovered.

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Introduction
The family Micropterigidae is currently (see, e.g. Kristensen et al. 2007) considered to have arisen in the first splitting event identifiable within the known extant Lepidoptera, i.e. it is the sister group of all other moths and butterflies. Representatives of the family are recorded from all zoogeographical regions, but the regional numbers of known species are highly uneven. This imbalance is almost certainly at least partly an artefact of inadequate sampling: micropterigids are small, very elusive moths with often very restricted flight seasons and they are normally obtained only through specialist collection, or by the use of automated devices such as Malaise traps which have but modest use in Lepidoptera inventories.

In terms of numbers of described species (77+), the Palearctic genus Micropterix Hübner, 1825 is at present by far the largest genus in the family (Zeller-Lukashort et al. 2007). Most Micropterix species occur in the Western Palearctic, in the Mediterranean subregion in particular and including Northern Africa, with only very few species ranges extending to the Eastern Palearctic. Just a single species (also that with the widest Palearctic distribution), Micropterix aureatella (Scopoli, 1763) is known to be present in the well investigated micropterigid fauna of the Japanese islands (Kozlov 1988; Hashimoto 2006).

Whilst almost all currently recognized Micropterix species are well characterized by distinctive features in the male genitalia, many are superficially similar overall, differing mainly in details in the forewing band-and-spot pattern (a few have unicolorous forewings) combined with cephalic (cranial and antennal cuticlevestiture) colouration. The female genital segments and cuticle-lined inner genitalia appear overall very uniform, and they have so far received little attention in a taxonomic context. However, recent observations revealed that females of some taxa do harbour variation that is taxonomically informative (Hashimoto 2006; Zeller-Lukashort et al. 2007).

Here, we describe a new clade containing two micropterigid species that are of outstanding interest because of their female postabdominal configuration, which deviates strongly from anything so far observed in the family. In spite of the moths’ phenetic uniqueness, also expressed in wing structure, we argue that they may best be considered a subordinate clade within the genus Micropterix Hübner, 1825, as might indeed be inferred from their general facies. We corroborate and expand these morphological arguments through the subsequent amplification and analysis of DNA barcode sequences (henceforth ‘barcodes’; Hebert et al. 2003) including several representatives of the genus Micropterix and two (DNA ‘mini-barcodes’ (Hajibabaei et al. 2006a) recovered from the two 75-year-old known specimens of one of the species and by examining their amino acid signature. Despite their potential utility for analysing museum specimens from otherwise unavailable taxa, the extent to which such short DNA sequences can assist taxonomic assignment has not been widely assessed (although see, e.g. Meusnier et al. 2008) and we provide some novel analyses, including distance and character-based approaches, that address this problem. The use of molecular character diagnostics (e.g. for identification of compact, taxonomically diagnostic combinatory motifs) has been recently advocated (e.g. Little & Stevenson 2007; Mikkola & Ståhls 2008; Rach et al. 2008; Prasad et al. 2009) but is still not widely implemented in barcoding studies.

The new clade is also of outstanding interest in a biogeographical context, being the southernmost known out-lier of the genus and the first representative from the Himalayan subregion. Inquiry into the history of finding of the specimens in question disclosed some information about the itineraries of the collectors (including an apparently unsung explorer, Mary Steele; see Supporting information), which appears relevant for assisting future searches for additional representatives of this structurally unique Micropterix clade and other primitive moths in this region.

Material and methods
Numbers preceded by ‘S’ refer to items in the Supporting information.

Specimen information
The allegedly Burmese moth specimens here treated were arranged under the genus Micropterix at the Natural History Museum, but remained undetermined since their acquisition (BMNH registration #1937-324). They were referred to by Kristensen & Nielsen (1979: 137) as females belonging to the M. aureatella group, hence whilst they were not then closely examined, both surely had the abdomen intact; however, it is now lacking from one. The Micropterix collection has been completely re-curated since that time (late G. S. Robinson, personal communication) and it is certain that no detached fragments are currently present in the relevant drawer (recently checked by DCL). The strikingly aberrant postabdominal configuration was noted, when the material was closely scrutinized by CZ-L and others acknowledged here (within the framework of an ongoing comprehensive study of the genus). Another specimen from India in the collection of the Berlin Museum für Naturkunde was brought to our attention by Dr Wolfram Mey in 2009. This specimen is a female with a similar (although not identical) postabdominal morphology.
A number of micropterigid lineages were selected for comparative purposes as part of a DNA barcoding program in the Barcode of Life Datasystems (BOLD: Ratnasingham & Hebert 2007, http://www.boldsystems.org/views/login.php). The taxon sampling is densest in the genus Micropterix of which 21 previously described species (≈27%) plus five additional putative new species (in addition to the new species treated here) were sequenced. Two species each of Epimartyria and Palaeomicroides, Auroterix sterops and two Trichoptera with full barcodes on GenBank representing fairly basal groups were used to root the tree; no obvious long-branch attraction artefacts were observed in tree runs. We refer to other GenBank and BOLD data accessible to us within the paper, including three basal homoneurous Lepidoptera. Pertinent data on the specimens examined are listed in Table 1. All the barcode sequences were selected to ensure a full overlap with the short sequences (mini-barcodes) obtained for the new Himalayan taxon, and only one species, Micropterix zangheriella Heath, 1963, shows a partial overlap (62.5%).

**Morphology**

The ‘lysed’ abdomen of the holotype of Micropterix cornuella (see Description; Fig. S9) lent itself to immediate examination/photography in glycerol and a permanent slide mount in euparal was eventually prepared; the Micropterix longicornuella holotype abdomen (Fig. 2E) was similarly treated after initial KOH maceration and staining in chlorazol black. The segment VIII ring of *M. cornuelle* had been ruptured probably during or after the extraction process, and fissures similarly developed in that of the fragile *M. longicornuella* holotype specimen upon KOH treatment; however, observations were not in any case critically impeded. For *M. cornuelle*, slide mounts were prepared of the most intact paratype fore wing and the holotype hind wings (which had come off together with the abdomen); all essential venational features of *M. longicornuella* could be observed (by transmitted light) without removal of the wings/scales. One of the intact antennae of the *M. cornuelle* holotype (Fig. S7) was lost after removal for photography; the other remains on the specimen, and the basal scale covering prevents a completely reliable flagellomere count.

**DNA extraction**

Tissue samples (a single dry leg) were pulled up from specimens with sterilized forceps, placed in 96-well plates, and shipped for processing at the Canadian Centre for DNA barcoding (CCDB). The only abdomen available for one of the two specimens preserved at the BMNH was processed by DCL using a minimally destructive protocol: the abdomen was first soaked in 20 μL of 20 mg/mL proteinase K solution mixed in Wizard® SV Lysis Buffer (containing 200 μL of Nuclei Lysis solution and 50 μL of 0.5 M EDTA) at 55 °C for 10 h, then genomic DNA was extracted using a Wizard® SV 96-well plate (both Promega, Madison, WI, USA) binding and elution system; the abdomen was then recovered for morphological examination, and the DNA extract was sent to Canada for amplification and sequencing (see below). At the CCDB, all other tissue samples were processed using the manual silica-based 96-well extraction protocol described in Ivanova *et al.* (2006). This protocol is ‘non’-destructive (see Porco *et al.* 2010) and careful recovery of the legs or leg fragments was carried out, demonstrating that high-throughput DNA extraction is compatible with tissue/voucher recovery.

**PCR amplification and sequencing of the barcode region**

Because the three Himalayan female specimens targeted here were collected 75–136 years ago, regular PCR amplification protocols used in Lepidoptera barcoding studies (e.g. Decaeńs & Rougerie 2008), which target fragments of c. 300–650 bp, are unlikely to yield any amplicon (Zimmermann *et al.* 2008). As a consequence, we attempted to amplify shorter fragments of 90–120 bp – ‘mini-barcodes’ (Hajibabaei *et al.* 2006a; Meusnier *et al.* 2008) – using six different primer sets developed for sphingid moths and already used with success to assemble full-length barcodes (658 bp) for archival (>130-year-old) specimens of geometer moths (Hausmann *et al.* 2009a,b). All other samples were processed with the usual Lepidoptera primer pairs: a first attempt with LepF1/LepR1 (Hebert *et al.* 2004) targeting a 658-bp fragment of COI, followed, for failures, with a second attempt with LepF1/MLepR1 and MLepF1/LepR1 (Hajibabaei *et al.* 2006b) targeting fragments of 307 and 407 bp.

PCR reactions were carried out in 12.5 μL reaction volumes containing: 2.5 mM MgCl₂, 1.25 μM of each primer, 50 μM dNTPs, 10 mM TrisHCl (pH 8.3), 50 mM KCl, 10–20 ng (1–2 μL) of genomic DNA and 0.3 U of Taq DNA polymerase (Platinum Taq DNA polymerase; Invitrogen, Burlington, Ontario, Canada). The thermocycling profile consisted of: one initial denaturation step of 1 min at 94 °C, followed by five cycles of 40 s at 94 °C, 40 s at 45 °C and 1 min at 72 °C, followed by 35 cycles of 40 s at 94 °C, 40 s at 51 °C and 1 min at 72 °C, with a final extension of 5 min at 72 °C. For mini-barcode fragments, we used the following touch-up profile (similar to Meusnier *et al.* 2008): a hot start for 2 min at 94 °C, followed by denaturation (40 s at 94 °C), annealing for 1 min at 46 °C, extension for 30 s at 72 °C, the last three steps cycled five times, then denaturation for 40 s at 94 °C, annealing for 1 min at 53 °C, extension for 30 s at 72 °C, the last three steps cycled 35 times, followed by a final extension for 30 s at 72 °C. PCR products were visualized on a 2% agarose...
Table 1 Details of specimens NHM = Natural History Museum, London; ZMUC = Zoological Museum University Copenhagen; MfN = Museum für Naturkunde, Berlin; RCDL = Research collection of DCL.

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D. C. Lees et al. • Morphologically unique micropterigid moths
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*Species provisionally identified preceding sampling of a range of barcodes; identities will be updated on the BOLD database in due course.*
E-Gel 96-well system (Invitrogen). Unpurified samples revealing faint to strong bands were cycle sequenced bidirectionally (with the same primers used for the PCR reactions) in 10 mL reaction volumes containing: 0.25 mL of BigDye v3.1, 1.875 mL of 5X ABI sequencing buffer, 5 mL of 10% trehalose, 1 mL of 10 µL primer, 0.875 mL of ultra-pure water and 1 mL of PCR product. The subsequent thermocycling profile was used for all products: initial denaturation at 96 °C for 2 min, followed by 30 cycles of 96 °C for 30 s, annealing at 55 °C for 15 s and extension at 60 °C for 4 min. Sequence reads were generated on an ATBI 3730xl DNA Analyser (Applied Biosystems, Foster City, California, USA) after clean-up with Sephadex (Sigma-Aldrich, Oakville, Ontario, Canada).

Editing, alignment and sequence repositories

After trimming exactly at the primer sequences, the trace files were edited and contigs assembled using Sequencher version 4.0.5 (Gene Codes, Ann Arbor, Mississippi, USA). The sequences obtained were clean and highly readable, with no double peaks or indels that could be suggestive of numts (Song et al. 2008). As a consequence, the final alignment using Bioedit version 7.05.3 (Hall 1999) was straightforward and non-ambiguous and we converted to the correct reading frame using a corrected Lepidopteran codon table (as in Linares et al. 2009).

All sequences generated and employed for this study are deposited both in GenBank and in BOLD, where specimen (including images) and sequence (including original electropherograms and primer information) data are publicly accessible.

Data analyses

Blast and distances. The sequences that were ‘blasted’ on GenBank using available algorithms (blatns, blastp, blastx) failed to provide an informative match at order level amongst returned amphibienopteran ‘hits’ (Supporting information, section 3). This is not surprising as a result of the complete lack of any public domain micropterigid COI data (see, e.g. Mikkelsen et al. 2007). Mini-barcode sequences obtained were therefore validated through a similarity matching test carried out using a local ‘blast’ applied using the Blastn algorithm in batch mode on a local database containing Micropterix sequences currently available to us on BOLD within the program Bioedit 7.05.3. P-distances and K2P distances were obtained using MEGA 4.02 (Tamura et al. 2007) and analysed in Excel simultaneously for 136 and 658 bp alignments containing the taxon set in Table 1.

Character diagnostics. Nucleotide positions are referred to from 1 to 658 of the standard barcode (equivalent to positions 1515 to 2172 of the Drosophila yakuba mitochondrial genome: Clary & Wolstenholme 1985) and amino acid positions are labelled correspondingly from 1 to 218 (i.e. from the first codon starting on the second nucleotide of the full barcode). Bioedit v. 7.0.5.3, in Conservation Plot mode, was used to visualize and analyse diagnostic characters. Comparative data from GenBank includes a few other trichopteran and lepidopteran sequences that are referred to in the text.

Parsimony analyses were run in TNT (Goloboff et al. 2008) using 1000 bootstraps, New Technology Search with all four options checked, and the most parsimonious tree(s) calculated with their length. Bootstrap values of at least 70% were considered ‘well-supported’.

Maximum likelihood estimates were run using PHYML v.3.0 (Guindon & Gascuel 2003). A GTR+I+gamma model was used (as determined in jModeltest: Posada 2008), using all possible options estimated, SPR swapping, and with 100 bootstraps. Values of at least 70% were considered ‘well-supported’ but values were reported where at least 50%.

Bayesian analyses were run using MrBayes 3.2 (Ronquist & Huelsenbeck 2003). A GTR+I+gamma model was also used, with first–second and third codons analysed under variable rates in separate partitions. One cold and three heated chains were run for 10 000 000 generations, a burn-in of 2500 samples, and after the average standard deviation of split frequencies had fallen below 0.01 (this occurred after 2 750 000–7 000 000 generations), whilst combining both runs which were checked for convergence using Excel. We used partitioning to account for rate bias as (1 + 2) and 3rd position codons treated separately. We also investigated elimination of noise from multiple transition hits using RY (purine-pyrimidine) coding of third positions (as implemented manually in MS Excel). Posterior probability (pp) support values of at least 0.95 were considered ‘well-supported’, but values are reported where at least 0.85.

Dating analyses A Bayesian analysis was separately run in BEAST v.1.4.8 and the resulting trees that were summarized in TreeAnnotator v.1.4.8, were visualized in FigTree v.1.3.1 (all programs from http://beast.bio.ed.ac.uk/), after a burn-in of 10%. Pp values were compared with those of MrBayes 3.2. BEAST was also used to provide a preliminary dating for the group, after an analysis run for 25 000 000 generations. Parameter statistics were checked in TRACER v.1.5 (http://beast.bio.ed.ac.uk/) with which it was determined that this run and thus the effective sample size for all parameters was sufficiently large. The dating was calibrated with a root height for the Amphibienoptera sensustricto (i.e. Lepidoptera + Trichoptera) of between 225 and 250 million years (hereafter Ma) (using a uniform distribution; initial value 240 Ma), based on known fossil history of this group (Grimaldi & Engel 2005, pp. 469, 553), and

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using a relaxed lognormal clock, under a GTR+I-gamma model with \([(1 + 2), 3]\) codons partitioned, and all parameters including base frequencies across codon positions unlinked. The tree prior was set to Speciation (Birth–Death process), with a UPGMA starting tree, and starting mutation rates were set very wide (0–0.025, coefficient of variation 0–0.0125), with other priors set to program defaults. Although there are many potential confounding biases in such an approach which include the interaction between the substitutional model used and its ability to deal with saturation (Papadopoulou et al. 2010), we checked the mutation rate resulting from this analysis, both by removing third positions and rerunning BEAST, and by applying a commonly reported substitutional rate for Lepidoptera/arachnids for an otherwise identical model, of 1.15% branch rate representing 2.3% pairwise divergence. Apart from the fossil history implicit in choice of root height, fossil data calibrations were not used due to lack of published data unequivocally demonstrating synapomorphies.

GIS
Georeferencing in Google Earth (standard version, 2008) enabled us to give considerable new precision to Mary Steele’s collecting data, based on the assumption that the date on the datalabels is accurate, and the elevations given in her logbook for that date are similarly correct (Fig. 1; Figs S1 and S2). Mary Steele’s itinerary was worked out by plotting these data on an overlay image from the US Army Corps of Engineers 1:250 000 China map ‘Li-Ma’ NH47-13, 1954, compiled from 1942 General Survey of India maps (http://www.lib.utexas.edu/maps/ams/china).

Results
Descriptive taxonomy
Genus Micropterix Hubner, 1825: M. cornella clade

Only females are known; the information on details of head and legs is based exclusively on M. cornella, but given the overall similarity between the two species included, the likely distinctive antennal traits are here ascribed tentatively to the clade.

Head. Cranium blackish, largely dull (microsculpture); ocelli present. Hair-scales bright orange-yellow, darkest on upper part of facial scale bed (Fig. S10). Antenna with \(\approx 37\) flagellomeres, basal \(\approx 13\) covered by lamellar scales [one antenna of the holotype of M. cornella (Fig. S7) was lost during removal for high-magnification photography; the other is left intact on specimen; both antennae of the M. cornella paratype were very incomplete (Fig. S10)]. Scape and pedicel orange-yellow; yellowish scale vestiture extending some distance distad on lower surface of flagellar base; flagellum and its vestiture otherwise blackish, hence in dorsal view markedly contrasting with scape and pedicel (Fig. 2D). Sensillum complement of antenna and maxillary palp not investigated.

Thorax. Fore tibial epiphysis originating near midlength and almost reaching tibial apex. Both wing pairs lanceolate, with pointed apex. Venation (Fig. 3) with fore wing Sc forked, R unforked and Rs twice dichotomously forked; Rs1 + Rs2 fork deep (stem \(\approx 0.5 \times R s1\)) and practically symmetrical; Rs3 and Rs4 ‘sessile’, but with common stem distinct basad from hyaline area around end of discal cell;

Fig. 1 Map showing currently known distribution along the Himalayas of the ‘cornella’-clade, country borders (in yellow). Square bull-eyes in yellow indicate type localities of Micropterix longicornuella and Micropterix cornella. White scale line, 500 km.
Rs4 terminating before/above apex. Hind wing Sc unfor-
ked, with moderate (anteriorly concave) subapical curva-
ture; no trace of a discrete R present; Rs as in fore wing; M-stem and bases of all M branches practically indiscern-
ible; CuP discernible only at base; single vein A tubular almost to wing margin. Fore wing (Fig. 2A–C) brownish-
purplish with golden basal and median fasciae, postmedian 
fascia incomplete, obsolete near dorsum; no subapical fas-
cia present (certainly true for M. longicornuella; scale vesti-
ture somewhat incompletely conserved in this wing region in both specimens of M. cornuella).

Pregenital abdomen. No trace of a sternum V gland pro-
tuberance could be observed. Tergal and sternal plates in 
mid-abdominal region (III–VII) overall uniform, near-rect-
angular (Fig. S9).

Female postabdomen (Figs 2E, 4 and 5). Tergum VIII 
and venter VIII plate fused, hence forming a complete 
sclerotized ring around the segment (Fig. 5A). The ring is 
least strongly sclerotized at the level of the upper margin 
of sternum VII, and anteriorly it is ± distinctly indented at 
this level. Segment IX with membranous dorsum and with 
strongly sclerotized and sharply delimited ventrolateral 
sclerotizations that are united in the midline by a short 
sclerotized bridge (Figs 4B and 5B). Each sclerotization 
bears a paramedial raised ridge, which posteriorly is pro-
duced into a prominent, setose and strongly melanized 
process. Terminal papillae (i.e. lateral plates on segment 
X) long, height somewhat diminishing posteriorly; greatest 
length (at mid-height) almost twice that of venter IX plate; posterior margin of papilla sclerotization almost straight. 
Each papilla (except most posterior region) very darkly 
melanized, particularly on dorsal part. Anteroventral part 
of papilla non-setose, with texture of minute, close-set and 
spine-bearing melanized protuberances; posterolateral 
papilla region with very few setae. Softwalled terminal 
abdominal rim flanking cloacal aperture as usually in micro-
pterigid moths with strong setae arising from elevated bases.
Ventral membrane just beyond sclerotized bridge between the right and left venter IX plates forming a protuberance; this protuberance, which is more pronounced in cornuella (Fig. 4A) than in longicornuella apparently has no relation to any internal structure (as far as could be ascertained on the basis of the material available).

Morphology-based diagnosis. It is the postabdomen, with the completely sclerotized segment VIII ring, the elongate, exceptionally shaped and strongly sclerotized ‘terminal papilla’ as well as the strongly sclerotized and sharply delimited laterventral IX plates with prominent paired processes, which set the M. cornuella clade markedly apart from all other micropterigid moths. In all so far examined members of the family tergum VIII is discrete and overall similar to the preceding terga, the segment IX sclerotization (often absent on the dorsum as in the M. cornuella group) never bears processes; a counterpart of the ventral membranous protuberance is absent, and the terminal papillae are always much less melanized, devoid of closest set and sclerotized spinose protuberances on anteroventral area, and posteriorly more evenly rounded. To our knowledge, these distinctive traits of the M. cornuella species group also have no counterparts in basal caddisflies or in other non-glossatan moths, hence they are surely autopomorphies of the group, whose monophyly is thus very strongly supported. The wing shape and venation render the known species of the M. cornuella group phenetically unique amongst micropterigid moths. No other members of the family known to us have the combination of distinctly lanceolate rather than rounded wings, all Rs branches in both wing pairs terminating in front of/above the wing apex, and the complete lack of a discrete vein R in the hind wing.

In forewing pattern and colouration (Fig. 2A–C), the M. cornuella-group species are overall similar not only to the widespread M. aureatella, but also to several other members of that genus including Micropterix myrtetella Zeller 1850, Micropterix aglaella (Duponchel, 1840), Micropterix aureoviridella (Höfner, 1898), Micropterix ibericella Caradja, 1920, Micropterix maschukella Alphéraki, 1870, and the SE European Micropterix wockei Staudinger, 1870 which (as noted below) in our preliminary molecular analyses consistently falls outside all other sampled congeners. The strong contrast between the bright orange-yellow antennal base with the blackish dorsal/lateral/medial flagellum surface and vestiture is unusual; in most other similarly banded/spotted Micropterix species, the entire antenna is blackish, but a pale scale vestiture does occur on the basal antennomeres (including basal flagellomeres) in some taxa;
Micropterix allionella (Fabricius, 1794), Micropterix hartigi Heath, 1981, and Micropterix trinacriella Kurz, Zeller & Kurz, 1997 are examples. The extension of lamellar scaling beyond flagellomere 10 is also uncommon in the genus.

**Molecular diagnosis.** See ‘Molecular data analyses’ section.

Micropterix cornuella sp. n. (Figs 1, 2A, B and D, 4; Figs S7–S10).

**Holotype.** ♀ ‘BURMA: Mishmi, Lohit River, 24.iii.1935, M.Steele’ [verbatim label text]. The type locality is clearly in the Mishmi Hills, Tellu [=Lohit] River, in present-day India (Arunachal Pradesh), probably near Krongma above Plawton at around 1700 m; see Table 1 and Supporting information (Figs S1 and S2). Museum voucher number: BMNH(E) # 732345; BOLD Barcode Sample ID: BMNH-MIC0002. Genomic DNA from lysed abdominal contents, BMNH(E) plate# MSL160: well H11. Genitalia slide N. P. Kristensen 1052.

**Paratype.** ♂: metathorax and abdomen missing, same data as holotype. Museum voucher number: BMNH(E) # 732448; BOLD Sample ID: BMNH-MIC0001.

**Etymology.** Alluding to the ‘horns’ on the venter IX sclerotization.

**Diagnosis.** At present with certainty distinguishable from M. longicornuella only by the female segment IX configuration, the relative short ‘horns’ being particularly distinctive (Fig. 4).

**Description.** See also preceding clade account.

**Thorax.** Fore wing length 3.5–3.8 mm, length/width ratio ≈2.9 (holotype) – 3.2 (paratype); postmedian fascia extending from costa (here very narrow) to M2 (here considerably widened).

**Female postabdomen.** Venter IX sclerite with process markedly bent relative to ventral margin of sclerite in front of process base; process length equal to this anterior sclerite length. Sclerite above process with few (≈10) setae (judged on the basis of alveole numbers). Spinose protuberances on anteroventral area of terminal papilla with spines near-parallel, backwards pointing.

**Mini-barcodes.** The mini-barcodes we obtained for the two Mishmi hills samples are 136 bp long. Two base differences confirmed for both specimens on either strand are shown in underlined bold below. Three autapomorphies for this species unknown in any of 26 other Micropterix species are shown in italics. Two different changes inferred to be from T->A that are shared uniquely amongst the Micropterix species examined with Micropterix tunbergella (Fabricius, 1787) and Micropterix calthella (Linnaeus, 1761), in respective order, are shown underlined only.

**Holotype:** GATTTCTTCAATTTTAGGGCTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT...
Morphologically unique micropterigid moths • D. C. Les et al.

Etymology. Alluding to the ‘horns’ on the venter IX sclerotization being relatively markedly longer than those of M. cornuella.

Diagnosis. At present distinguishable from M. cornuella with certainty only by the distinctive female genitalia; for other differences see under Description.

Description. Single available specimen very incomplete: antennae, fore- and mid legs as well as left fore wing in front of Cu1b lacking; right forewing glued to mesothorax, and any remains of the prothorax and head are concealed in the glue (Fig. S6). Overall very similar to M. cornuella.

Wings. Forewing length ≈3.7 mm, perhaps relatively slightly broader (length/width ratio ≈2.5, but accurate length measurement impossible because of incompleteness of wing base) than in M. cornuella. Basal (in particular) as well as postmedian fascia somewhat broader, and median fascia with inner margin more concave than in available M. cornuella, but the taxonomic significance of these small differences evidently cannot be assessed on the basis of a single specimen.

Female postabdomen. Venter IX sclerite (Figs 2E and 5) much larger and with process considerably more prominent than in M. cornuella, process not markedly bent relative to ventral margin of sclerite in front of process base; process length more than twice this anterior sclerite length. It remains uncertain whether the divergence of the processes in the untreated specimen (Fig. 2E) is a desiccation artefact, or their parallel arrangement in the KOH-treated specimen (Fig. 5B) is a preparation artefact; the latter seems most likely. Sclerite above process with marked, oblique strengthening ridge paralleling posterior margin, and with many more (≈30) setae. Spinose protuberances on anteroventral area of terminal papilla not all backwards pointing, lower group arranged in whirl-like fashion.

Mini-barcode. The same approach as the one used for the two samples of the above described M. cornuella failed to recover any sequence data from the single very old specimen available.

Molecular data analyses

PCR. The PCR yielded a faint band at the expected fragment size exclusive of primers, 136 bp, for one of the six primer pairs used [COI_bc_SphF5 (5′-ATTTTTTTCGCT-TCATTTRGCTTGG-3′) and COI_bc_SphR5 (5′-GTAA- TTGCTCCTGCTAATCTGG-3′)].

Distance analyses

Pairwise divergence of the two sequences. The sequences of the holotype and paratype are 136 bp long and differ in two bases that could be clearly called in either direction (i.e. are 1.47% pairwise divergent). This precise level of divergence in other Micropterix species for the sequenced region always (n = 18 comparisons) underestimates that in the full barcode, where it equates to 1.5–5.5% divergence (Fig. 6). Divergence values over about 2.7–3% have been considered typical of interspecific divergences elsewhere in the Lepidoptera or elsewhere, whereas intraspecific divergence may typically be an order of magnitude lower (Hebert et al. 2003), whilst it is now clear that interspecific divergences may descend as low as 0%, leaving no barcoding gap (Meyer & Paulay 2005).

Blast. Blast searches using a range of algorithms on GenBank are summarized in Supporting information (section 3). The local blast analysis based on nucleotides (implementing the algorithm blastn), used either mini-barcode of M. cornuella as query sequence. This yielded a top hit of Micropterix berytella, closely followed by an unidentified species from Samos, Micropterix aruncella (Scopoli, 1763), Micropterix klimeschi Heath 1973, Micropterix elegans Stainton, 1876, Micropterix berytella de Joannis, 1886 and M. sp cf. berytella, as the seven top congeneric but allospecific hits, at modest pairwise divergence (p-distance) and also K2P levels of between 4.4% and 5.3% (Table 2). Despite the two bases’ difference between the holotype and paratype sequence, the ranking of blast hits and scores is almost identical (Table 2). As the local Blast procedure automatically excluded five of the 136 bp for allospecific hits, the actual pairwise divergence for p-distance and K2P distance for the full 136 bp was also calculated (Table 2, last two columns).

Fig. 6 Pairwise (p-) distances for Micropterix–Micropterix and all other informative comparisons (i.e. Micropterix cornuella excluded) showing how distances for the full barcode in other species relate to distances at the sequenced region, for identical species comparisons.
Table 2 Local Blast hits and rankings (using Blastn) for the nucleotide sequences BMNH-MIC0002 (HT) and BMNH-MIC0001 (PT) (using all sequences referred to in Table 1 as the local database), along with pairwise and K2P divergences calculated using MEGA4. HT = Holotype; PT = Paratype.

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<th>Score (bits)</th>
<th>Expect value</th>
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PT: rank order of hits when PT used as query; in bold; results thus identical for PT. *37.5% overlap for one sequence, otherwise 100% (this short sequence was ranked 31st by blastn but is placed in its approximate position based on p-distance).

The high COI divergences, even within the family Micropterigidae for this region, are evident, whilst differences with Micropterix are relatively modest. The divergences with the sequenced region are also strongly correlated with average p-distances within the entire barcode, as calculated using Mega ($r^2 = 0.963$) but this does not mean they are necessarily representative, especially at low divergence levels (Fig. 6). The variation observed means that, although there is also a correlation in such cases (for comparisons within Micropterix excluding cases involving M. cornuella which are uninformative, $r^2 = 0.73$), we cannot be confident that a distance method has recovered the closest match, were the entire barcode known. Thus, for Micropterix comparisons, six base pairs’ difference (that observed to M. calthella for M. cornuella) at 136 bp (i.e. 4.4% p-distance) equates to between 21 and 55 bp difference (i.e. 3.2–8.4% p-distance) for the whole 658 bp barcode (Fig. 6). This variation implies a lack of robustness in the likely ranking of hits as more of the barcode is sequenced; there can hardly be a species more different to M. cornuella in wing pattern than the all-gold M. calthella and hence their DNA similarity might indeed be misleading. The observed tendency of the sequenced 136 bp fragment to underestimate the level of divergence for the complete barcode region (Fig. 6) is, however, consistent with it being a relatively conserved region of the barcode.

Although less informative than nucleotides in terms of similarity, the 45-codon long amino acid sequence of...
M. cornuella (ISSILGAINFISTIMNPKIYLNLFNQIPLFVWSVKITALLLLSL.I) is compatible with it being not only a micropterigid, but probably a taxon subordinate within Micropterix. Of the 26 thus far sequenced Micropterix species on BOLD that were available to us (Table 1), 16 taxa have an identical amino acid sequence to M. cornuella (namely, M. berytella, M. sp cf. berytella, M. elegans, M. klimeschi, M. aruncella, M. sp. (Sarnos), M. turnbergella, Micropterix aureoviridella, Micropterix sicanella Zeller, 1847, M. sp. (Aquadolce), Micropterix culturensis Heath, 1981, Micropterix schaefferi Heath, 1975, M. sp. (Vizinni), M. aureatella, M. calthella, Micropterix islamella Amsel, 1935) one has a shorter sequence that cannot be distinguished (M. zanghieriella), whereas six species differ by a single codon (Micropterix rubensis Zeller, 1868, M. trinacriella, M. myrytetella, M. allionella, M. bartigi and Micropterix erectella Walsingham, 1919). Micropterix wockei differs by two codons, M. sp. (Monte Cassino) by at least two, and Micropterix rotbenhachii Frey, 1856 by four codons. By comparison, the sequence difference to two other Northern hemisphere micropterigid genera examined here is 20–22.2%, i.e. nine sequence difference to two other Northern hemisphere rothenbachii M. sp. (Monte Cassino) by at least two, and

**Character diagnostics**

Character diagnosis provides significant additional insight for the current dataset to a distance method, at the same time bypassing a ‘black box’ approach common in molecular systematics. It is fortuitous that the region sequenced includes one of the most taxonomically informative parts of the barcode. The conserved/non-saturated nature of several codons within this sequence is likely connected with the fact that this fragment lies largely within the adjacent fourth and fifth helices rather than in ‘loop’ parts of the molecule (for secondary structure of COI, see, e.g. Tsukihara et al. 1996). In purely practical terms, codon characters are much more convenient for pattern recognition and potentially far more locally unique than nucleotides and are therefore considered here in this light. In particular, the COI amino acid sequence of all known Micropterix is so distinctive from that of any other currently sequenced insect genus accessible to us and to all sequenced Micropterigidae belonging to other genera (unpublished data on BOLD), that there is little room for doubt that the sequences of the Mishmi Hills individuals also belong to Micropterix.

A highly diagnostic 16-codon string can be recognized in the middle part of the fragment (that lies entirely within the fifth helix along COI in the 5’ direction) of both specimens: ‘YNLNFNQIPLFVWSVK’. The positional sequence motif ‘YN’ (tyrosine, asparagine) commencing this sequence is rare amongst Amphiesmenoptera, occurring in Micropterix and in a handful of austral micropterigid (Tasmanix and Zealandopterix zonodacta; unpublished data on BOLD), the glossomatid caddis flies Glossosoma nigrior (AF436493) and Anagapetus debilis (AF 436497) and the ecnomid Ato psyche sp. (AF436591). The same character combination also recurs very rarely amongst the many thousands of other lepidopterans so far barcoded that are publicly accessible on GenBank or BOLD: e.g. the heliozclid Coptodisc a kalmiella (AF150907), the coleophorid Coleophora versurella (MECB971-05) and the arctiid Spilosoma congrua (LGSC711-05DN-ATBI-2711), with none of which is the character state likely to be homologous. The final codon ‘K’ (lysine), is diagnostic of Micropterix within Micropterigidae but very rarely occurs elsewhere in the Amphiasmenoptera (it is either putatively plesiomorphic or it recurs in Heterobathmia and several basal ecomid, stenoscyld and hydrobiosid caddisflies, or has evolved as a parallelism in a very few lepidopteran genera such as Argyresthia in the Yponomeutidae; GenBank and BOLD). This lysine change thus represents one of eight potentially locally unique and unreversed amino acid changes for Micropterix along the barcode for the present dataset (Fig. S3) Combined, these three positional characters may uniquely distinguish Micropterix. Furthermore, the short motif ‘FN’ (phenylalanine, asparagine) within this string is private to the genus Micropterix amongst all barcoded Micropterigidae except M. wockei (thus already representing the majority of known family diversity: unpublished data on BOLD).

Variability at amino acid level for potentially uninformative unreversed characters within the barcode for the present dataset is summarized in Fig. S3. Whilst most of the potentially informative characters are at generic level or above, three character changes, an M->L in position 112, an N->K in position 119, and an I->V in position 165 (at least the last change not represented in M. cornuella), appear to be attributes of particular groupings with Micropterix (Fig. S3). At present levels of taxon sampling in the genus, it is early to draw conclusions about the taxonomic utility of these changes, considering also they may have arisen in parallel within the genus as...
inferred by particular phylogenetic trees (see below), but they are flagged for future work. With regard to a possible internal position of *M. cornuella* within *Micropterix*, the most important of the potential changes is that from YD -> FN in the 162–163rd codon in all *Micropterix* except *M. wockei*.

Apart from non-synonymous changes implied above, two nucleotide characters within the dataset deserve mention, even though both represent third positional T->A changes, as they are uniquely shared in the two 75-year-old specimens of *M. cornuella* amongst the 26 presently barcoded *Micropterix* species with *M. tunbergella* and a species from Samos (450th nucleotide) and with *M. calthella* [546th nucleotide of the barcode (see mini-barcodes under Description)].

**Phylogenetic analyses**

All northern hemisphere taxa here treated (Table 1; Fig. S3) were always fully supported as monophyletic, as were the component genera *Palacramoecidae*, *Epimartyria* and *Micropterix*. As expected from the foregoing analyses, *M. wockei* fell basally in all phylogenetic and also distance-based analyses, usually subtended by a relatively long branch to other species, which is, however, not quite as long as that for *M. cornuella*. The subterminal grouping of *Micropterix* that excluded *M. wockei* was not always supported (improval of support values on removal of up to four short sequences suggests this due to ‘floating’ of such sequences), and its topology varied between analyses, i.e. was unresolved. Both *M. cornuella* sequences grouped together (except if they were run in a 136 bp alignment) as did at least three distal groups that were supported in all analyses. The precise placement of *M. cornuella* is thus still tentative, also considering its short sequence.

We present here (Figs 7 and 8) two topologies based on Bayesian inference (one using a BEAST dating) as a framework for briefly summarizing here the results of the different analyses (for brevity not all trees are shown but see Figs S4 and S5). In all phylogenetic analyses [parsimony based (TNT) on new technology search, maximum likelihood (ML) based on PHYLML, and bayesian, based on MrBayes (MB) and BEAST], in addition to *M. cornuella*, the three groups amongst *Micropterix* always recovered as monophyletic consisted of *M. berytella*, *M. cf. berytella* and *M. elegans* (hereafter referred to as the ‘*M. elegans*-group’, a group that never included *M. islamella*; see also Zeller-Lukashort et al. 2009), a clade consisting of *M. cornuella* and *M. sp. (Vizinni)* and a clade consisting of *M. sicanella* and *M. sp. (Aquadolce)*. Thus, only closely related species groups, and groups near or above generic level, were supported by the barcode data. Nevertheless, many of the sister groupings shown in trees here (Figs 7 and 8; Figs S4 and S5) agree with earlier suggestions of affinities amongst species (Heath 1973, 1981a,b).

**Placement of *M. cornuella***

 Parsimony. Using TNT, under a New Technology search with default options (tree not shown), four most parsimonious trees were found of length 782, three of these with *M. cornuella* placed next to *M. tunbergella* or to *M. tunbergella + M. sp* (Samos), and one next to *M. calthella* and in turn sister to *M. sp* (Monte Cassino) and then to *M. tunbergella + M. sp* (Samos). Such groupings failed to be well supported and the consensus of these trees was a bush with the above usually recovered clades, plus *M. trinacriella + M. myrtetella* and *M. schaefferi + M. vulturensis*. The ingroup excluding *M. wockei* was also not well supported in cladistic analyses.

ML. The PHYLML analysis placed *M. cornuella* as sister of the *M. elegans* group, without support (Fig. S4). The Micropterix ingroup excluding *M. wockei* was also not recovered as well supported in ML analyses, even when the six shortest sequences (including *M. cornuella*) were removed (Fig. S4). When the four shortest sequences (not including *M. cornuella*) were removed for an RY coded dataset, bootstrap support for the ingroup was modest at 54%.

MB. The MrBayes analysis partitioned into nucleotides [(1 + 2) + 3] positioned *M. cornuella* at the root of a grouping of (*M. aruncella + M. klimeschi*) + the *M. elegans* group, but threshold support levels for this placement were also not achieved. When the apparently ‘floating’ sequence of *M. zangberiella* (262 bp) was removed, the Micropterix ingroup that excludes *M. wockei* was well supported (pp = 0.97; tree not shown), as for when the four shortest sequences excluding *M. cornuella* were removed for an RY coded dataset (pp = 1.0; Fig. S5).

**Dating analyses**

BEAST. In the BEAST dating analysis, *M. cornuella* was grouped with *M. tunbergella + M. sp. (Samos)* or with *M. sp. (Samos)* alone (Fig. 8). Well supported groupings included *M. allionella + M. zangberiella + M. vulturensis + M. schaefferi* in addition to *M. elegans + M. cf. berytella*, and all three taxa in *M. elegans* group (Figs 7 and 8).

With the ‘root height’ age at 236.8 Ma, the origin of Micropterigidae appeared to be relatively ancient (161.3 Ma; with wide variation, 95% Highest Posterior Density or HPD: 90.9–231.5 Ma; values from Tracer), with the basic split in northern hemisphere taxa at 76.9 Ma (95% HPD: 37.3–123.0 Ma), whilst the crown group radiation of *Micropterix* appeared to be relatively
recent (25.3 Ma; 95% HPD: 9.9–44.9 Ma); with mean figures, but not HPD, highly variable between BEAST runs. The ingroup excluding M. wockei had a nodal age of 18.1 Ma (95% HPD: 7.7–31.2 Ma). The two other Northern hemisphere genera diverged around 38 Ma (95% HPD 13–57 Ma), with the branch to the Micropterix crown group thus about 13 Ma longer. Variation in the M. cornuella population we sampled here could on this basis be interpreted to have arisen in the last 0.83 Ma (with an implied branch substitution rate of 1.24%), with MRCA of the Himalayan group going back c. 7.9 Ma, dependent on its phylogenetic placement close to Micropterix sp. (Samos), or back to 18.1 Ma (95% HPD: 7.7–31.2 Ma), dependent on a (better, but still not well supported: pp = 0.85) subordinate position within the ingroup excluding M. wockei. The TMRCA of the genus Palaeomicroides appears to be somewhat more recent than that of Micropterix (12.5 Ma; 95% HPD: 2–16 Ma), as for that of Epimartyria 6.7 Ma (95% HPD 3–22.4 Ma). The wide error bars are probably a reasonable reflection of the uncertainties in this dating exercise that might be fine-tuned in future using fossil data, which incidentally the range of values from BEAST does not appear to contradict. It may be noted that this result also implies a quite low mean rate of per site substitution pMa in the barcode region (from BEAST, around 0.809%, about 70% of one more typically quoted branch substitution rate for this marker (c. 1.15% or 2.3% pairwise divergence) reported generally in the literature for arthropods or even other Lepidoptera: Papadopoulou et al. 2010). The mean substitution rate from BEAST was over an order of magnitude lower (0.058% branch rate) for first and second positions alone. With a mean branch mutation rate for all positions set thus to 0.015, the time of most recent common ancestor (TMRCA) of Amphiesmenoptera was inferred to be 135 Ma, Micropterigidae 94 Ma, that for the northern hemisphere lineage at 37 Ma and that for Micropterix at 15.5 Ma, all much more recent datings than the fossil his-
Fig. 8 Dating and topology using BEAST with a partitioned analysis with (1st + 2nd) and 3rd positions coded separately. Posterior probability support values are shown in Fig. 7. Scale is set to a root height of 240 Ma. Error bars in blue are 95% HPD confidence limits. *Micropterix cornuella* is shown in red.

tories of these groups, and for Trichoptera, even the lower limit of the 95% HPD for the TMRCA being 162 Ma, whereas even the fossil history of Rhyacophilidae goes back further (Grimaldi & Engel 2005).

**Discussion**

**Systematic affinities and age of the Micropterix cornuella group**

The completely unique configuration of the female post-abdominal segments of the two species described above might *a priori* seem to indicate that the new taxa occupy a very isolated position within the family Micropterigidae. However, the clearly preapical position of vein Rs4 in both wings is a *Micropterix* autapomorphy. Moreover, a suite of other apomorphies of these taxa shared at least partially with other micropterigid taxa are all similarly present in, and characteristic of, *Micropterix*; these include: The pronouncedly pointed-lanceolate forewings, which are paralleled within the S. African *Agrionynpha* and some SW Pacific taxa; the unforked forewing R vein, paralleled also in all other N. Hemisphere genera, the Australian *Australomartyria porphyrodes* and an undescribed Malagasy genus; the absence of any trace of a sternum V gland (shared with S. American *Hypomartyria* and some undescribed Malagasy species; interestingly, the latter are subordinate members of a genus in which most members do have prominent gland protuberances).

The single and simple vein in front of the hindwing Rs is a noteworthy difference from the condition so far ascribed to *Micropterix*, viz., the plesiomorphic retention of fully developed veins Sc and R, subapically united by what was long considered a cross-vein, but which may just as well be an Sc2 (and the apical portion of apparent R hence an anastomosis product; Kristensen & Nielsen 1982). But whilst unique amongst *Micropterix* species, the simplified condition may obviously well have been evolved within the genus. This is paralleled in some members of the Nearctic/East Asian genus group (*Epimartyria*, some *Neomicropteryx* and...
Isikiomartyria; Issiki 1931; Hashimoto 2006), some Australasian lineages including Tasmantrix calliplaca (Meyrick, 1902), Z. zonodoxa (Meyrick, 1888) and A. sterope (Turner, 1921) (see Gibbs 2010), the S. American Squamicoria and the afore-mentioned undescribed Malagasy genus.

The above-mentioned traits are thus compatible with the Mishmi Hills/Darjeeling clade being closest relatives of Micropterix. But moreover, and most importantly, morphology provides no evidence to support the monophyly of Micropterix excluding this clade, which could, therefore, conceivably have a subordinate position within the genus, its phenetic uniqueness notwithstanding. However, the presently available morphological information cannot per se bring the analysis any further.

The morphology of the specialized processes on females of these two new species do, however, deserve a final remark. We call attention to parallel cases of moth species with highly aberrant/autapomorphic postabdominal morphology being otherwise unexceptional in their generic context; the heterobathmiid Heterobathmia valcifer is an eye-opening example (Kristensen & Nielsen 1998; Kristensen 2003: 113).

Further insight is provided by the molecular analyses based on barcodes. The results of our character-based, phylogenetic and dating analyses, although unresolved for the placement of this Himalayan clade, suggest that the M. cornuella group is probably subordinate within the bulk of the genus. This last clade may have originated only around 18 (7.5–31) Ma, and is inferred to have split from M. wockei about 25 (10–45) Ma, and so the M. cornuella group is probably much more recent than 31 Ma. Without the benefit of molecular data, the predominantly western Palearctic distribution pattern of Micropterix led Corley (2007) to suppose that Micropterix constitutes a relatively recent radiation of micropterigid moths. We have revealed a penetration of this genus though the Himalayas, but our analyses confirm the recent nature of the radiation of extant Micropterix. This result implies a low average rate of substitution in the Micropterigidae for COI of 0.8% per site per million years, comparable to that found for the family Papilionidae COI–COII by Zakharov et al. (2004). If a slightly higher substitutional rate typical for Lepidoptera/arthropods, e.g. 1.15% pMa were applied, all TMRCs would be far too young for known fossil history. The rather slow ‘clock’ that we infer here for Micropterigidae is a matter for future analyses to confirm, preferably using an appropriate range of genes and a higher taxon sampling density. If Baltic amber fossils dated at around 37 Ma indeed belong to the crown group of Micropterix, an even slower clock would be implied for Micropterix.

Molecular analyses are consistent in placing M. cornuella not only internal to the sometimes well supported ingroup of Micropterix inferred for this dataset to be sister to M. wockei, but in a fairly distal position amongst the eight of the 16 other species in our analysis that possess an identical amino acid sequence. Both Bayesian methods and a few most parsimonious trees favour a placement close to M. tunbergella and an undescribed Greek species from Samos, the latter of which ranks almost as high as M. calthella in our local Blast ranking, rather than within a middle eastern ‘M. elegans’ group (see also Zeller-Lukashort et al. 2009) or next to an (M. arnecella–M. klimeschii) + M. elegans group, as was suggested by our ML analysis.

Given that the COI marker is rarely dedicated for phylogenetic reconstruction at least on its own, it is entirely unsurprising that a relatively short fragment of COI provides no further resolution, and to be expected that if further fragments can be obtained, they might provide a more robust indication of the sister taxon of the Himalayan clade. However, our analysis paves the way for more rigorous tests of the potential of mini-barcodes from hitherto unknown taxa such as those in museums to provide taxonomic assignment. In our case, it lends doubt that future work will prove the new cornuella-clade to be basal within Micropterix, let alone sister to it. The addition of other markers capable of resolving a relatively recent divergence in relation to the Cretaceous fossil history of Micropterigidae, is of course highly desirable in future work, as it is further clear that even the whole barcode based on nucleotides or their translation, provides too few data to generate a reasonably well resolved tree, at least based on existing levels of taxon sampling.

These molecular results are nevertheless consistent with the female postabdominal modifications being indeed highly derived structures that characterize a subordinate Micropterix lineage, and thus caution against describing taxa with bizarre morphology in new genera. From its morphology, M. cornuella is clearly the presently known sister to M. longicornuella, but further resolution must await additional material from the Himalayan region, including the male sex and immatures, and a phylogenetic systematization of the many described Micropterix species, preferably using a combination of molecular and morphological methods.

On the distribution range of Micropterix

Micropterix is one of the rare Lepidopteran genera to benefit from a fossil record, with one fragmentary wing impression fossil ascribed to this genus by Jarzembowski 1980 from the Oligocene of Isle of Wight, one Baltic amber species (Kurz and Kurz, 2010) and a number of undescribed specimens that may with greater certainty be placed in the genus preserved in Baltic and Saxonian amber (Kristensen & Skalski 2010).
Oriental region by six other genera (Gibbs et al. c. are known from Burma preserved in much older and beautifully preserved micropterigid fossils presence of members in eastern Siberia. Although several not managed to negotiate the Bering landbridge, despite the and exclusively Palearctic radiation of the genus, that has consistent with our dating, that would suggest a post-KT dispersal or vicariance) only by lineage represented in the Americas (whether as a result of dispersal or vicariance) only by Epimartyria and in the Oriental region by six other genera (Gibbs et al. 2004; Hashimoto 2006).

The fact that one of the new taxa here described have turned up in old material procured by a generalist collector (Mary Steele; details in Supporting information) of mostly zoological specimens is suggestive that the genus is undersampled in the southeast of its range and there may exist a substantial fauna of Micropterix following, if not ranging just outside, the southern limit of the Palearctic Region, usually defined to include the foothills of the Himalayas above 2000 m. It is therefore apparent that inventory efforts for primitive Lepidoptera should be greatly stepped up in workable countries of this region, using appropriate modern collection methods including day searching, sweeping and malaise trapping. Indeed, the genus has also recently been detected in the far eastern Himalayas, in Yunnan, China (Y. Kobayashi, personal communication). Serious efforts should also be made to discover the family Micropterigidae in the ‘Deccan trap refugia’ of SW India and Sri Lanka, given the recent discovery of this family in Madagascar by one of us (DCL). In that case, it might be anticipated that any Micropterigi- dae surviving in the southern Indian region do not belong to the putative ‘northern hemisphere’ lineage, as is supported from COI data for the new northern Indian taxa reported here.

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References


Supporting Information
Additional Supporting Information may be found in the online version of this article:

Appendix S1. Biographic notes for collectors, type locality details, supplementary figures and references and blast hits on Genbank.

Fig. S1. Itineraries of Mary Steele in 1935 and 1936.

Fig. S2. Detail from inset rectangle in Fig. S1.

Fig. S3. Summary of potentially informative amino acid changes along the COI DNA barcode.

Fig. S4. Tree resulting from ML analysis.

Fig. S5. Tree resulting from Bayesian analysis with RY-coding.

Fig. S6. Micropterix longicornuella holotype female before detachment of left hindleg.

Fig. S7. Micropterix cornuella holotype female before abdomen detachment.

Fig. S8. M. cornuella holotype abdomen before lysis.

Fig. S9. M. cornuella holotype abdomen after lysis.

Fig. S10. M. cornuella paratype specimen before leg removal.

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