

Taxonomic problems in the subgenus *Meloehalea* Wirth of the genus *Atrichopogon* Kieffer (Diptera: Ceratopogonidae) inferred from both morphological and molecular characters

Andrea Tóthová, Jan Knoz, Radim Sonnek, Josef Bryja & Jaromír Vaňhara

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The classification of *Meloehalea* (Ceratopogonidae) species is based on morphological characters. The taxonomic revision of generally well-known species recovered interesting facts on possible geographic intraspecific variability or on the presence of an additional valid species. In this study, 5 European species and 2 species from the U.S.A. and Canada have been compared based on their morphological characters. Subsequently, the 16S rDNA sequences analyses of well known European species have been performed to confirm the characters mentioned in the determination key of this subgenus. Although, this study was limited by the number of examined non-European specimens, it provides some interesting facts on the possible morphological variability of well-known species and also an initial phylogenetic backbone for the progressive reconstruction of infrageneric relationships within the genus *Atrichopogon*.

A. Tóthová, R. Sonnek, J. Vaňhara, Masaryk University, Faculty of Science, Department of Botany and Zoology, Kotlářská 2, 611 37 Brno, Czech Republic; A. Tóthová's e-mail: tothova@sci.muni.cz

J. Knoz, Masaryk University, Faculty of Science, Department of Experimental Biology, Kotlářská 2, 611 37 Brno, Czech Republic

J. Bryja, Department of Vertebrate Biology, Academy of Sciences CR, 675 02 Studenec 122, Czech Republic; Masaryk University, Faculty of Science, Institute of Botany and Zoology, Kotlářská 2, 611 37 Brno, Czech Republic

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1. Introduction

The subgenus *Meloehalea* Wirth of the genus *Atrichopogon* Kieffer contains a group of biting midges that suck haemolymph from Meloidae and related beetles. This subgenus currently includes 16 species (Szadziewski & Borkent 2004, Remm 1988, Wirth 1980) of which 12 spp. occur in the Palaearctic and 7 spp. in the Nearctic re-

gion. These species are usually distinguished on the basis of morphological characters such as TR (tarsal ratio), number of mandible teeth, proboscis section length etc. (Wirth 1956, 1980, Szadziewski *et al.* 1995).

During an extensive biomonitoring study in North-western Bohemia (Bílina Area) and in Southern Moravia (Podyjí National Park) in the Czech Republic (1997–1999 and 2001–2003)

that was focused on most dipteran families, we obtained material of three biting midge species of the subgenus *Melochelea*, i.e. *Atrichopogon (Melochelea) winnertzi* Goetghebuer, 1922, *Atrichopogon (Melochelea) lucorum* (Meigen, 1818) and *Atrichopogon (Melochelea) oedemerrarum* Storå, 1939. Based on a morphometric analysis several additional specimens were identified as *A. (M.) epicautae* Wirth, 1956 following the key by Wirth (1980). Our material from Central Europe was morphologically compared with the type material of *A. (M.) epicautae* and additional specimens of *A. (M.) lucorum* from the U.S. National Museum of Natural History (USNM) (Washington), the Canadian National Collection (Ottawa) and from prof. William Grogan (Salisbury University, Maryland). The American species *A. (M.) downesi* Wirth, 1980 and *A. (M.) farri* Wirth, 1956 are clearly characterised and are not included in this study, which also applies to *A. (M.) atriscapulus* Kieffer, 1918. Due to the recent knowledge, we consider both species *A. (M.) winnertzi* Goetghebuer, 1922 and *A. (M.) meloesugans* Kieffer, 1922 as valid (Szadziewski, pers. comm.).

Subsequently, we performed a DNA analysis comparing all Czech species of *Melochelea* using DNA sequences of mitochondrial 16S rDNA. The problems with species identification together with enormous practical importance of the group necessitate more accurate tools for the classification and identification of biting midges (Gomulski *et al.* 2005, Pages & Monteys 2005). The use of molecular data to resolve taxonomical problems within the family has been very scarce and the published studies include almost exclusively the genus *Culicoides* Latreille, 1809 because of its medical importance (Sebastiani *et al.* 2001, Linton *et al.* 2002, Gomulski *et al.* 2005, 2006, Pages & Monteys 2005, Perrin *et al.* 2006). In these studies, RAPD, enzymatic restriction of the COI gene and ITS regions 1 and 2 were used for differentiation of sibling species complexes. The first two methods do not seem too reliable and credible to solve these taxonomic problems. Mitochondrial (16S, COI, cytB) and nuclear genes (ITS1) have been used to solve intrageneric taxonomic inconsistencies in other dipteran families, (Pestano *et al.* 2003, Krüger *et al.* 2000, Guryev *et al.* 2001) and it is reasonable to test the usefulness

of these markers also in biting midges.

In the only study focusing on the phylogenetic relationships within Ceratopogonidae based on molecular data, sequences of the *cox2* gene were analysed. These results supported the monophyly of the family as well as the recent general classification based on morphological characters (Beckenbach & Borkent 2003).

2. Material and methods

The specimens of own collection were mounted into microscope slides and examined using an Olympus BX51 microscope. The material was collected by various methods excluding attraction to light (for more details see Tóthová *et al.* 2004, 2005). Drawings and pictures were prepared using the same microscope with the SW MicroImage v. 4.0. For DNA analyses, the abdomen and thoracic musculature were removed for further processing and the remainder of each specimen was deposited as voucher specimen at the Department of Botany and Zoology (Masaryk University Brno, Czech Republic).

2.1. Specimens examined

The following characters were measured: AR – antennal ratio; CR – costal ratio, PR – palpal ratio, PR-N – palpal ratio used in this study, see below, R2/R1 – radial field ratio, TR – tarsal ratio.

2.1.1. *Atrichopogon (Melochelea) epicautae*, *sensu* Wirth, 1956

The following specimens were examined: 1–5, Paratype females; 6–8, females from Canada; 9–11, females from Arizona (see Table 1):

- 1–4, Rustlers Park, Chiricahua Mts., Arizona, 25.VI.1953, leg. Wirth, attacking *Meloe* beetles
- 5, Rustlers Park, Chiricahua Mts., Arizona, 25.VI.1953, leg. Wirth, attacking *Meloe* beetles (drawn and measured in Wirth 1980)
- 6, Kouchibouguac National Park, N.B., 10.VII.1978, coll. L. Forster, No. J.A.D. 1194/1/29

Table 1. Measurements of the females (1–11) of *Atrichopogon epicautae* examined. Abbreviations: AR: antennal ratio, CR: costal ratio, PR: palpal ratio, PR-N: palpal ratio used in this study, R2/R1: radial field ratio, Wing: wing length in mm, Mt: number of mandible teeth, TR: tarsal ratio, MuB: macrotrichia under the basal field of the wing.

	AR	CR	PR	PR-N	R2/R1	Wing	Mt	TR	MuB
1	1.8	0.68	2.75	5.12	2.27	1.38	19	2.29	no
2	1.82	0.66	2.66	4.11	2.41	1.29	18	2.24	no
3	2.17	0.66	2.76	5.29	2.30	1.38	17	2.28	no
4	1.97	0.65	2.63	4.63	2.82	1.34	18	2.31	no
5	2.09	0.65	2.9	4.46	2.90	1.4	16	2.14	no
6	2.04	0.68	2.85	3.3	2.85	1.18	19	2.22	yes
7	1.98	0.68	2.76	3.88	2.51	1.08	16	2.3	yes
8	1.94	0.7	2.66	3.89	2.76	1.2	18	2.44	yes
9	2.46	0.65	3.13	5.42	2.54	1.32	17	2.27	no
10	2.25	0.65	3.08	5.02	2.38	1.16	18	2.27	no
11	1.84	0.69	2.63	3.7	2.7	1.2	17	2.40	yes

- 7, Kouchibouguac National Park, N.B., 2–13.VII.1977, coll. J.R. Vockeroth, No. J.A.D. 1157/2/8
- 8, White face Mt., N.Y., 79-506, 10.VII.1979, coll. D.F.J. Hilton, No. J.A.D. 1237/1/4
- 9, Yavapai Co. Ariz., A77-61, Red Rock Crossing, 19.VII.1977, M.W. Sanderson LT, leg. Wirth
- 10, Coconino Co. Ariz., A77-82 Oak Creek, Cumphouse Wash., 26.VIII.1977, M.W. Sanderson, BLT, leg. Wirth
- 11, Cranberry L., N.Y., St. Lawrence Co., 25.VI.1963, swamp, leg. Wirth.

2.1.2. *Atrichopogon (Meloehalea) lucorum* (Meigen, 1818)

The following specimens were examined: 1–5, females from Smithsonian Inst.; 6–8, females from Canada; 9–10, females from Maryland, USA; 11–14, females of other European species of the subgenus *Meloehalea* (see Table 2):

- 1, England, Wylam Northumberland, N. bank Tyne, 6.V.1960, leg. D.A. Humphries, attacking *Meloe* beetles
- 2, Scotland, Aberlady Bay, 4. VI. 1964,

Table 2. Females of *Atrichopogon lucorum* (1–10) and other European species of the subgenus *Meloehalea* examined. 1–10: *A. lucorum*, 11: *A. "epicautae"*, 12: *A. meloesugans*, 13: *A. oedemerarum*, 14: *A. orbicularis*, 15: *A. winnertzi*. Abbreviations as in Table 1.

	AR	CR	PR	PR-N	R1/R2	Wing	Mt	TR	MuB
1	2.76	0.78	3.3	5.84	3.24	1.41	22	2.6	yes
2	2.74	0.7	2.99	5.33	3.6	1.52	23	2.46	yes
3	2.49	0.66	3.04	5.7	3.22	1.41	17	2.25	no
4	2.71	0.7	3.01	4.84	3.24	1.43	19	2.4	yes
5	2.87	0.68	3.14	5.64	3.02	1.6	20	2.36	yes
6	1.97	0.7	4.09	5.68	2.33	1.53	23	2.4	4
7	2.28	0.66	3.9	5.67	2.25	1.55	30	2.44	4
8	2.3	0.67	3.8	5.80	2.3	1.51	26	2.3	no
9	2.21	0.73	2.94	5.23	2.71	1.3	21	2.64	yes
10	2.17	0.69	3.17	5.40	3.16	1.28	19	2.48	yes
11	2.76	0.7	2.7	4.97	2.96	1.40	18	2.29	3
12	1.8	0.66	3.9	5.63	2.54	1.45	25	2.48	3
13	1.6	0.7	3.61	5.62	2.84	1.53	16	2.57	yes
14	2.45	0.74	3.46	5.63	2.5	1.73	27	2.38	yes
15	2.97	0.7	4.27	8.94	2.31	1.50	36	2.57	yes

Table 3. Collection sites and GenBank sequence accession numbers of biting midges used in the DNA analyses in this study.

Species	Collected in	16S
<i>Dasyhelea saxicola</i> Edwards, 1929	Podyjí NP, Czech Republic	DQ364965
<i>Atrichopogon lucorum</i> 1 Meigen, 1818	Podyjí NP, Czech Republic	DQ364959
<i>Atrichopogon lucorum</i> 2 Meigen, 1818	Šumava NP, Czech Republic	DQ364960
<i>Atrichopogon lucorum</i> 3 Meigen, 1818	Šumava NP, Czech Republic	DQ364961
<i>Atrichopogon lucorum</i> 4 Meigen, 1818	Šumava NP, Czech Republic	DQ364962
<i>Atrichopogon lucorum</i> 5 Meigen, 1818	Jizerské hory PLA, Czech Republic	EF472587
<i>Atrichopogon</i> aff. <i>epicaetae</i> 1	Podyjí NP, Czech Republic	DQ364958
<i>Atrichopogon</i> aff. <i>epicaetae</i> 2	Podyjí NP, Czech Republic	DQ364964
<i>Atrichopogon winnertzi</i> Goetghebuer, 1922	Podyjí NP, Czech Republic	DQ364957
<i>Atrichopogon oedemerarum</i> 1 Storå, 1939	Podyjí NP, Czech Republic	DQ364966
<i>Atrichopogon oedemerarum</i> 2 Storå, 1939	Podyjí NP, Czech Republic	DQ364963

- Abdullah, R. & E. Crowson, coll. at cantharidin
- 3, Arizona, Rustlers Park, Chiricahua Mts., 11.VII.1958, O'Brien & Ross, light trap
 - 4, England, Wylam Northumberland, N. bank Tyne, 6.V. 1960, leg. D.A. Humphries, attacking *Meloe* beetles
 - 5, Scotland, Gale, 9.VI.1964, R. & E. Crowson, coll. at cantharidin
 - 6, Glacier Cr., B.C., 10 m, N. Terrace, 2.VI.1960, coll. W.W. Moss, No. J.A.D. 1238/5/2
 - 7, Glacier Cr., B.C., 10 m, N. Terrace, 2.VI.1960, coll. W.W. Moss, No. J.A.D. 1238/5/1
 - 8, Shames B.C., 18 m SW. Terrace, 23.VI.1960, coll. C.H. Mann, No. J.A.D. 1238/4/1
 - 9, Maryland, Prince George Co. Patuxent Wildlife Refuge, 19.V.1979, malaise trap, leg. Wirth
 - 10, Maryland, Prince George Co. Patuxent Wildlife Refuge, 23.V.1979, malaise trap, leg. Wirth
 - 11, *A. "epicaetae"*, Podyjí NP, Czech Republic, 31.V.–1.VI.2001, car net, leg. Barták, coll. Tóthová
 - 12, *A. meloesugans*, Wyskok, Poland, 1.VI.2003, leg. Szadziewski, coll. Tóthová
 - 13, *A. oedemerarum*, Bílina Area, Czech Republic, XI.1998–III.1999, malaise trap, leg. Barták, coll. Tóthová
 - 14, *A. orbicularis*, Bieszczady, Poland, 22.VI.2004, leg. Gwizdalska, coll. Tóthová

- 15, *A. winnertzi*, Podyjí NP, Czech Republic, 26.VI.–24.VII.2001, malaise trap, leg. Barták, coll. Tóthová.

2.2. DNA Extraction

All the material used for DNA analysis (Table 3) was preserved in 95% ethanol. Individual flies or tissue portions were ground up in absolute ethanol, dried and rinsed in deionized water and placed in sterile Eppendorf tubes with extraction buffer (60 mM EDTA, 0.2% SDS, 10 mM Tris-HCL, pH=8) and pulverised in liquid nitrogen. After adding proteinase K, samples were incubated at 56°C overnight. The DNA was extracted first with phenol:chloroform:isoamyl alcohol (25:24:1) and again with chloroform:isoamyl alcohol (24:1). From the obtained solution, the DNA was finally extracted with the QIAQuick PCR Purification Kit (QIAGEN) following the manufacturer's protocol.

2.3. Molecular analysis

Modified primers mt32 (5'-CAACATCGAG-GTCGC-3') and mt34 (5'-TTGACCGTGCA-AAGGTAG-3') (Nirmala *et al.* 2001) were used for both amplification and sequencing the ca. 320bp long fragment of the mitochondrial 16S rRNA gene. Amplifications were performed in a 20ul reaction mixture containing 1× PCR buffer (Fermentas), 2mM MgCl₂, 1.2U Taq polymerase

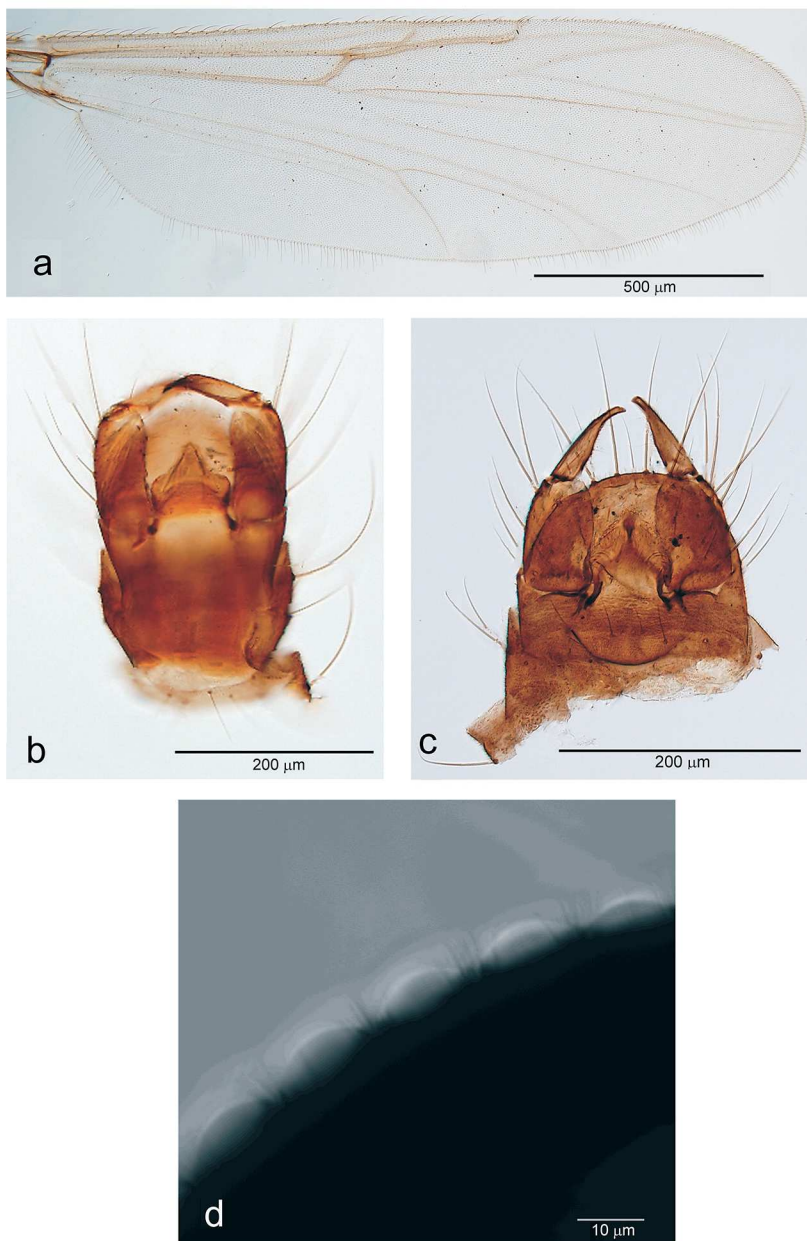


Fig. 1. Males identified by Wirth as *A. epicaudatae*. – a. Wing of the male drawn by Wirth (1980). – b. Hypopygium of the same specimen. – c. Hypopygium of a male from the same collection. – d. Infuscated eyes of the specimen drawn by Wirth (1980).

(Fermentas) and 4µM of each primer. Temperature cycling generally consisted of a 2 min initial denaturation at 94°C, followed by 35–38 cycles including 94°C for 30 s, 53°C for 30 s and 72°C for 1 min 30 s and final extension at 72°C for 7 min. PCR reactions were performed in Robocycler Gradient 96 thermal cycler (Stratagene). All PCR products were visualized via agarose gel electrophoresis to assure proper amplification and detect

possible contamination using negative controls. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) and used directly for sequencing. The sequencing reactions were performed in a 10µl reaction mixture using the Big Dye Terminator v. 1.1. After the thermocycling, the reactions were purified by the EDTA/ethanol precipitation before the injection to the ABI310 Genetic Analyser (Applied

Biosystems). Sequences were determined for both strands of the PCR product.

Species *Dasyhelea saxicola* (Edwards, 1929) from the sister tribe Dasyheleinae was used as out-group to root the phylogenetic trees (Table 3).

2.4. Alignment and phylogenetic analysis

Sequences of both strands were edited using Sequencher v. 4.5 (GeneCodes) and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.* 2004) and PAUP v.4.0b10 (Swofford 2002). The alignment was performed using ClustalW incorporated in MEGA v. 3.1 with the default settings. Phylogenetic relationships were reconstructed using maximum parsimony (MP), neighbour-joining with HKY85 substitution model (NJ) and maximum likelihood (ML) methods. Maximum parsimony heuristic searches for optimum trees were performed by swapping branches using the tree bisection reconnection (TBR) algorithm for 500 replicates, with all substitutions given equal weight and gaps treated as missing characters. Branch support was assessed by 1,000 bootstrap replicates. The ModelTest 3.8 on-line tool (Posada 2006) was used for testing and choosing the best model for likelihood analysis. The obtained trees were edited in TreeView (Page 1996) and the final layout was performed using Adobe Photoshop 8.0 CS.

3. Results

3.1. Morphological characters

Females of the subgenus *Melohelea* have two seminal capsules, bare eyes and a short proboscis that is straight or bent anteriorly. The sensory pit on the third palpal segment is usually located at midlength. The proximal antennal flagellomeres are broad and disciform, closely appressed and the teeth of the mandibles are largest at mid-porch.

Males have a more or less uniformly coloured scutum. Their proboscis is shorter than head height, straight or bent anteriorly. A sensory pit is

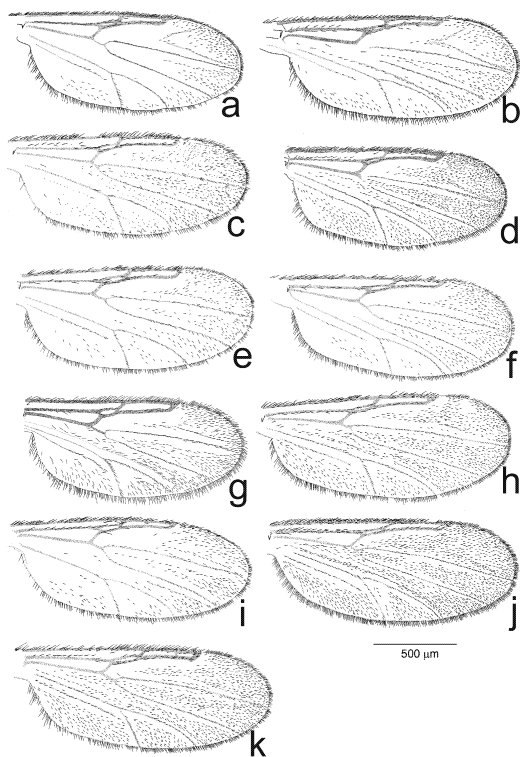


Fig. 2. Wings of the specimens of the subgenus *Melohelea* examined. – a. *A. epicautae* paratype. – b. *A. epicautae* from Canada (Kouchibouguac National Park). – c. Specimen from the Czech Republic identified as *A. epicautae*. – d. *A. lucorum* from Scotland. – e. *A. lucorum* from Canada (Glacier Creek, B.C.). – f. *A. lucorum* from Canada (Shames B.C.). – g. *A. lucorum* from Maryland, USA. – h. *A. oedemerarum* from the Czech Republic. – i. *A. meloesugans* from Poland. – j. *A. orbicularis* from Poland. – k. *A. winnertzi* from the Czech Republic.

located at midlength or near to the apex of the third palpal segment. Genitalia are not enlarged, as broad as the tip of the abdomen, gonostylus with simple pointed apex. Wing membrane is with macrotrichia, eyes bare. Interesting is the fact, that the male of *Atrichopogon* (*Melohelea*) *epicautae*, described by Wirth (1956), has pubescent eyes and its hypopygium is very similar to those of the subgenus *Atrichopogon*. This is probably a missidentification and male specimens described as *A. (M.) epicautae* sensu Wirth (1980) need to be re-evaluated. For example, two males identified as *A. (M.) epicautae* on loan of the Smithsonian Institution are not similar. Both

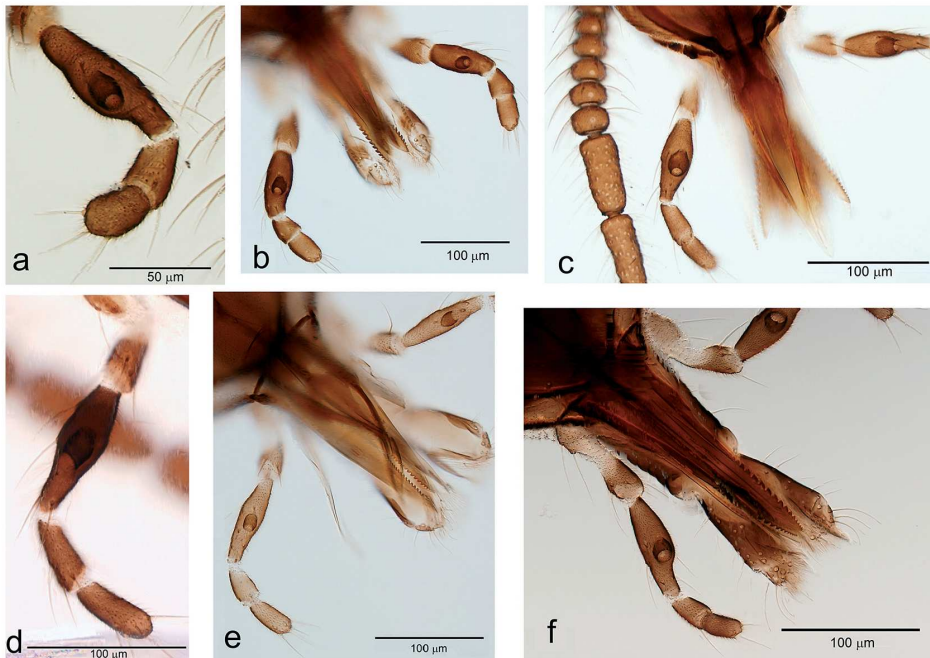


Fig. 3. Palps and mouthparts of examined specimens of the subgenus *Meloehalea*. – a. *A. epicautae* paratype. – b. *A. epicautae* from Canada (Kouchibouguac National Park). – c. Specimen from the Czech Republic identified as *A. epicautae*. – d. *A. lucorum* from Scotland. – e. *A. lucorum* from Canada (Glacier B.C.). – f. *A. lucorum* from Maryland, USA.

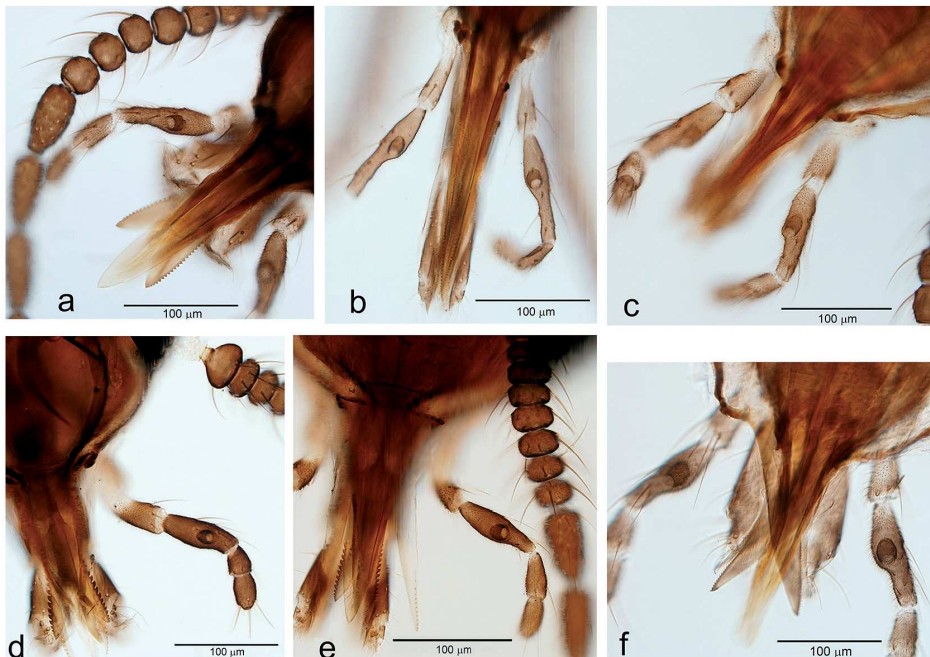


Fig. 4. Palps and mouthparts of examined specimens of the subgenus *Meloehalea*. – a. *A. meloesugans* from Poland. – b. *A. winnertzi* from the Czech Republic. – c. *A. oedemerarum* from the Czech Republic. – d. *A. epicautae* from Cranberry Lake, N.Y., St. Lawrence Co. – e. *A. lucorum* from Arizona. – f. *A. orbicularis* from Poland.

males have pubescent eyes, bare wings, but their genitalia are different (see Fig. 1a–d). The second radial cell is $1.78\times$ and $2.5\times$ (the specimen drawn by W.W. Wirth) longer than the first radial cell and the fifth palpal segment is rounded apically. All these characters are typical of the subgenus *Atrichopogon* (Szadziewski et al. 1995).

The analysis of the morphological characters of the *Meloehalea* specimens revealed several interesting facts.

- 1) The study of the paratype females of *A. epicautae* allows us to define their distinctive characters: absence of macrotrichia under B (see Fig. 2), R2/R1 ratio (up to 2.90) and the ratio of the length of the 3rd palpal segment and its width on the distal third of the segment – PR-N ratio (4.11–5.29).
- 2) Canadian specimens of *A. epicautae* have many macrotrichia under the basal field (B), and PR-N = 3.3–3.89, which is on the border with those of *A. lucorum* and paratypes of *A. epicautae* (see Figs 2–4).
- 3) The specimen from the Czech Republic identified as *A. epicautae* indicates very close relation to this species following Wirth's key. It has no macrotrichia under B and the R2/R1 ratio is 2.96. However, the 303bp of 16S rRNA gene sequence of this specimen was completely identical with those of *A. lucorum* from the Czech Republic. The identification of *A. epicautae* based on published characters is thus questionable.
- 4) The *A. epicautae* specimen no. 11 from Cranberry Lake, St. Lawrence Co. New York also lacks the typical characters of *A. epicautae*; it has numerous macrotrichia under B (see Fig. 5a–b) and the shape of the 3rd palpal segment is different of that of *A. epicautae* paratype (Fig. 6). That poses another question about the validity of this character, which might be subject to intraspecific variability. In other *Meloehalea* species the presence and abundance of macrotrichia on the wings appears to be a stable character.
- 5) The majority of material from the Smithsonian Inst. came from Europe (England, Scotland and Estonia). All of them were typical *A. lucorum* with numerous macrotrichia under B, TR = 2.34–2.6, and PR-N = 4.84–5.84. The

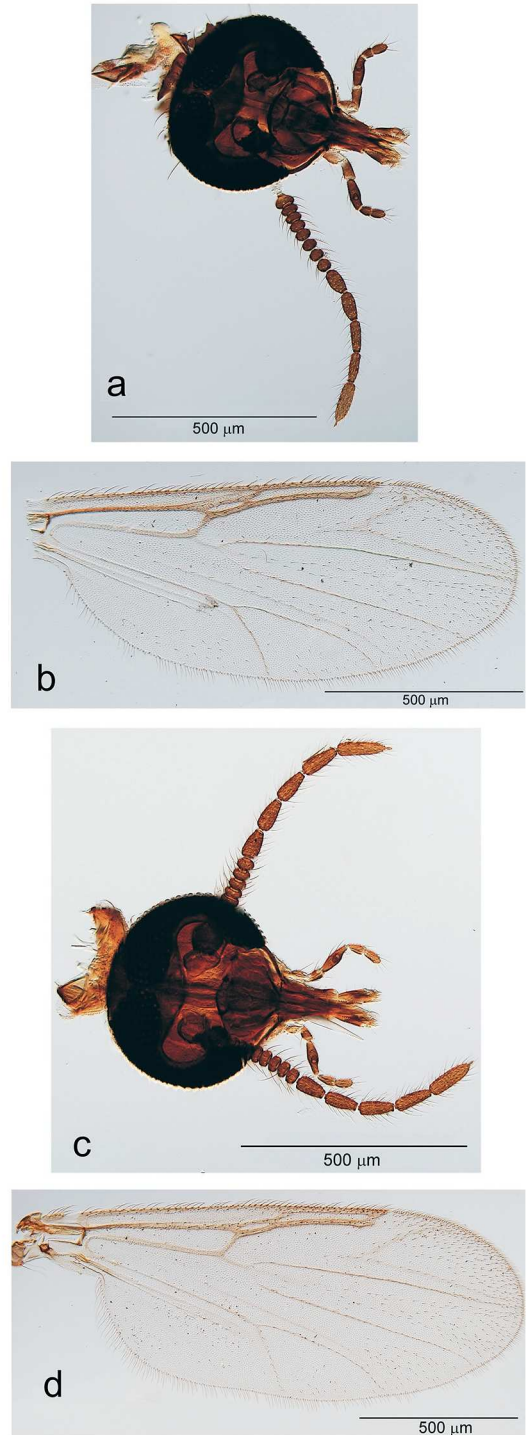


Fig. 5. Doubtfull specimens. – a. and b. *A. epicautae* (Cranberry Lake, N.Y., St. Lawrence Co.). – c. and d. *A. lucorum* (Arizona).

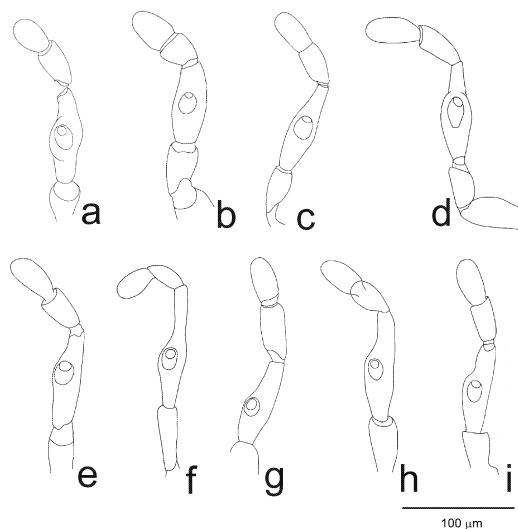


Fig. 6. Palps of examined specimens of the subgenus *Meloehalea*. – a. *A. epicautae* paratype. – b. *A. epicautae* from Canada (Kouchibouguac National Park). – c. Specimen from the Czech Republic identified as *A. epicautae*. – d. *A. lucorum* from Scotland. – e. *A. lucorum* from Canada (Shames B.C.). – f. *A. winnertzi* from the Czech Republic. – g. *A. meloesugans* from Poland. – h. *A. oedemerarum* from the Czech Republic. – i. *A. orbicularis* from Poland.

specimen collected in the U.S.A. (Arizona) identified as *A. lucorum* had no macrotrichia under B (see Fig. 5c–d); however, on the basis of measurements alone, it is very close to *A. lucorum*. Similarly, the status of the Canadian *A. lucorum* is not clear. It has a PR= 4, inferring a relation to *A. winnertzi*. However, if we take PR-N (5.64–5.68) the Canadian specimens of *A. lucorum* fit in the typical “*lucorum*” group. This Canadian species has mandible teeth very similar to those of *A. winnertzi* (see Fig. 7), but has no (or only few) macrotrichia under B on the wing, which is another difference between *A. lucorum* and *A. winnertzi*.

- 6) There is no type male of *A. epicautae*. The male specimens collected and described by Wirth (1980) do not belong to the subgenus *Meloehalea*, as they have bare wings, infuscated eyes and their hypopygia are not similar (see Fig. 1a–d).

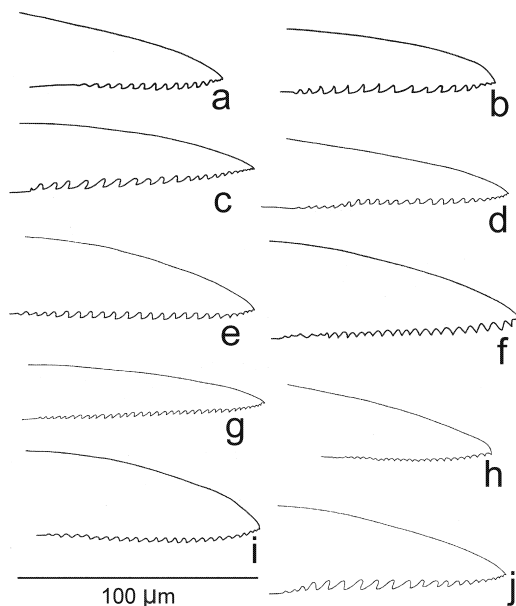


Fig. 7. Mandible teeth of examined specimens of the subgenus *Meloehalea*. – a. *A. epicautae* paratype. – b. *A. epicautae* from Canada (Kouchibouguac National Park). – c. *A. lucorum* from Scotland. – d. *A. lucorum* from Canada (Glacier Creek, B.C.). – e. *A. lucorum* from Canada (Shames B.C.). – f. *A. oedemerarum* from the Czech Republic. – g. *A. winnertzi* from the Czech Republic. – h. *A. orbicularis* from Poland. – i. *A. meloesugans* from Poland. – j. Specimen from the Czech Republic identified as *A. epicautae*.

3.2. Analysis of 16S rDNA sequences

The length of the 16S rDNA resulting alignment was of 303 bp of which 222 were constant and 31 parsimony-informative. The alignment is available upon request from the senior author. By maximum parsimony PAUP found the single most parsimonious tree shown in Fig. 8. The hierarchical likelihood ratio test (IRT) as implemented in Modeltest 3.8 (online tool) suggested the use of the F81+G model for maximum likelihood analysis as optimal for this dataset (assumed nucleotide frequencies A=0.41610, C=0.05560, G=0.10530 T=0.42300, rates=gamma, shape=0.2072, $-\ln L=726.18163$, pinvar=0) with 4 rates categories and a 50% majority – rule consensus tree was performed (see Fig. 8). The topologies of all obtained trees were similar, only the different bootstrap values are shown above and below the

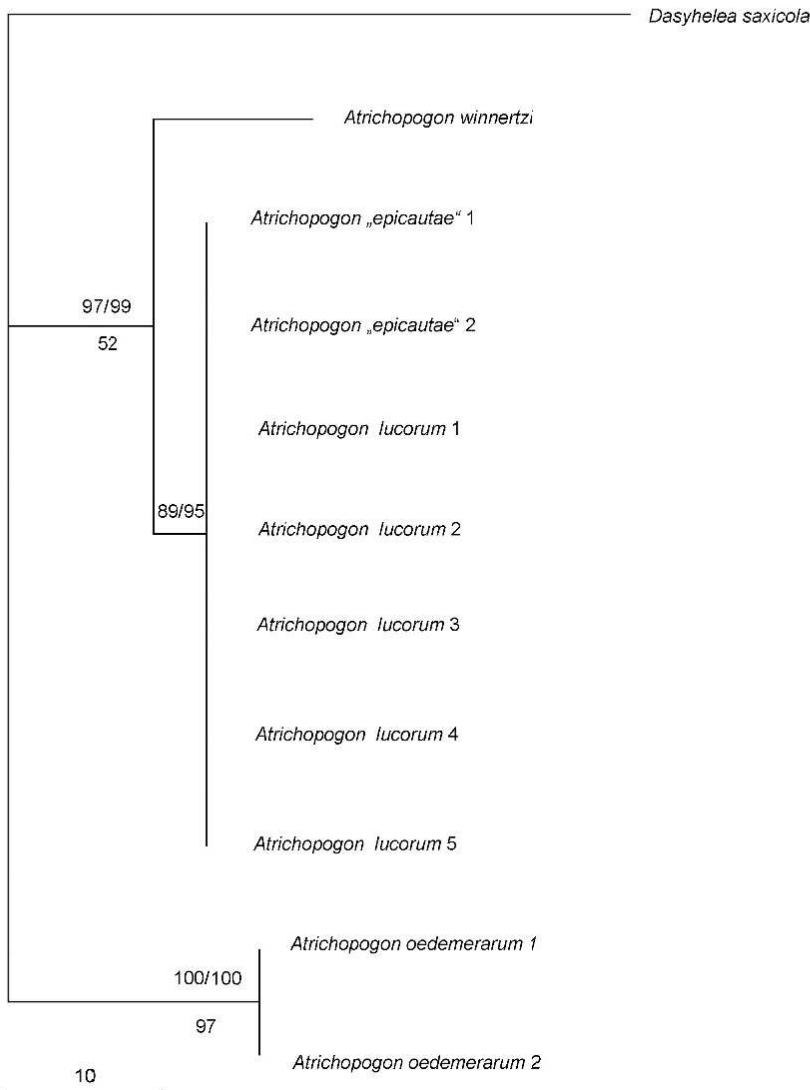


Fig. 8. Maximum parsimony tree for the included biting midge taxa obtained by analysing 16S rDNA data set; the length=94, CI excluding uninformative characters=0.9524, RI=0.9565, RC=09362. Bootstrap values are shown above (maximum parsimony/neighbor-joining) and below (maximum likelihood) the branches (1000 replicates). Scale: 10 substitutions/site.

branches. The pairwise distances using the HKY85 substitution model are shown in Table 4. The minimum evolution score of the NJ tree was 0.34910. The interspecific variability of *Meloehalea* species with distance values varied between 5.6–14.5%. All of the obtained phylogenetic trees fully correspond with the recent systematics of the subgenus *Meloehalea* based on the form of the proximal flagellomeres (Wirth 1980), however the analysed specimens of *A. (M.) lucorum* and *A. (M.) „epicautae“* were identical (pairwise-distance 0.00% among these two species). This result supports the usefulness of the 16S gene on lower taxonomic levels. It means

also the suitability of this morphological character mentioned above (shape of the proximal flagellomeres) in separation of main groups of the subgenus *Meloehalea* and incorrectness of the characters used for differentiation of species *A. (M.) lucorum* and *A. (M.) „epicautae“* (hind tarsal ratio, proboscis section length ratio). The pairwise distances support these facts, as well. The species *A. oedemerarum* is separated from the “*lucorum*” group by virtue of its moniliform flagellomeres; *A. winnertzi* is related to *A. lucorum* by their shared disciform flagellomeres but separated due to the number and shape of mandible teeth and PR (PR-N). The sequences of the

Table 4. Pairwise distances of 16S rDNA sequences using HKY85 substitution model.

	<i>D. saxicola</i>	<i>A. winnertzi</i>	<i>A. lucorum</i>	<i>A. oedemerarum</i>
<i>D. saxicola</i>	–	–	–	–
<i>A. winnertzi</i>	0.28487	–	–	–
<i>A. lucorum</i>	0.23850	0.05611	–	–
<i>A. oedemerarum</i>	0.24668	0.14450	0.11471	–
" <i>A. epicaudae</i> "	0.23850	0.05611	0.00000	0.11471

mitochondrial 16S gene of all analysed specimens of *A. "epicaudae"* and *A. lucorum* were identical, so we did not detect any geographical variation in these. However, the characters used in the key by Wirth are doubtful and the segregation of these two species is probably incorrect. For the exact identification of the species in the subgenus *Meloehalea* it will be necessary to discover other characters that are not subject to intra- or interspecific variation (e.g. presence/absence of macrotrichia on the wing, R2/R1 ratio, PR-N).

4. Discussion

We did examine the holotype of *A. (M.) epicaudae* (type no. 62405 U.S.N.M) as well. Unfortunately, it was a pinned female specimen, so the characters used in this study were not available. Therefore we refer to the paratype females only. We documented no consistent pattern in both the presence of macrotrichia and morphometric characters frequently used for species identification in the subgenus *Meloehalea*. However, the specimens of the type series of *A. epicaudae* and the typical specimens of *A. lucorum* from Scotland and Maryland have distinctive characters, which allow defining these species (macrotrichia under B, shape of the 3rd palpal segment, R2/R1 ratio, PR-N). Characters as CR, wing length, antennal ratio, shape of spermatheca (similar in all mentioned species) are not informative in the process of species determination. The problematic specimens of both species from Canada and of *A. lucorum* from Arizona could be the result of intraspecific variability. However, in case of the Canadian *A. lucorum*, it is probably a misidentification and these specimens represent a new species (their mandible teeth are completely different from those both of *A. lucorum* and *A. epicaudae*).

The separation of *A. lucorum* and *A. epicaudae* from other *Meloehalea* species is supported by the shape of the proximal flagellomeres, PR (PR-N) and the shape and number of mandible teeth.

All these results pose the following questions: (1) Are all of the above-mentioned species really valid taxonomic units or only evidence of extreme morphological variation within a single species? (2) Are there valid differences between European and Canadian (American) *A. lucorum*? The answer to these questions will require more material for morphological and genetic investigations as well as more data on the biology and distribution of this species. It is also necessary to discover the male of *A. epicaudae* (if the validity of this species will be supported despite the intraspecific variability) and to include it into the type series.

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