# Danish *Culicoides* species of the Obsoletus – group identified by morphological methods

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According to Campbell and Pelham-Clinton (1960), Kremer (1965) and other authors is it difficult to separate females of the subgenus Avarita (Obsoletus group) especially the females of *C. obsoletus* and *C. scoticus*.

In a quotation from Baldet et al., 2008 "For some species, like *C. obsoletus* and *C. scoticus* both belongig to the Obsoletus group, females cannot be separated morphologically".

The aim of our study is to show that it in fact is possible to separate the four species of the Obsoletus group by combining a few morphological characters.

### **Material and Methods**

### Field sampling

The biting midges were collected (light traps) at two localities in Middle and Southern Sweden and from more localities on Zealand, Denmark

The insect material was transferred to 70% ethanol. Prior to the DNA measurements the *Culicoides* specimens were morphologically identified under a stereomicroscope.

### Identification

- > Males
  - Hypopygium
  - One wing

- > Females
  - Head
  - One wing
  - Posterior abdominal segments

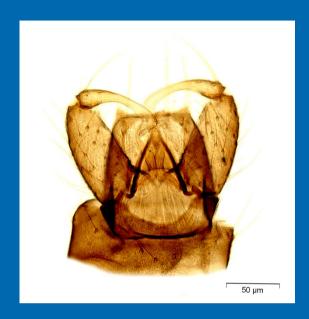
Slide mounts were made.

The remaining parts were transferred to 96 % ethanol in a test tubes for DNA analysis.

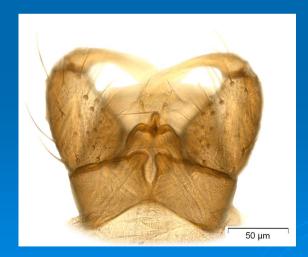
### Measurements

- Lengths of every palpal segments
- Lengths of flagellar segments
- Lengths of wings (from arculus to tip)
- > Lengths of spermatheka
- Antennae ratio (AR: 11-15 antennal segments divided with segments 3-10)
- Palpal ratio (PR: length of segment 3 divided with greatest breath)

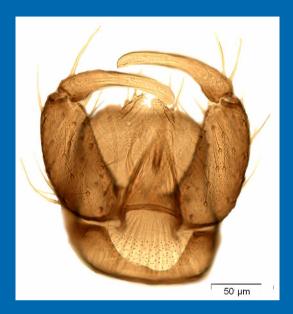
### Hypopygium



C. chiopterus



C. obsoletus

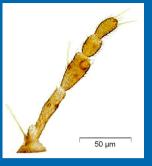


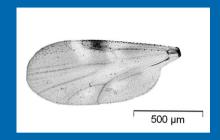
C. dewulfi

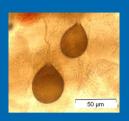


C. scoticus

### Females – Obsoletus group

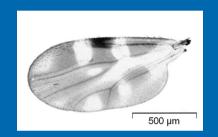


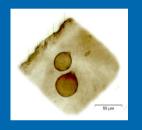




C. chiopterus

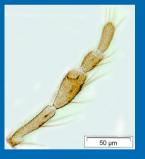


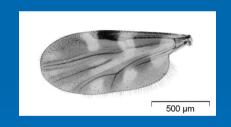




C. dewulfi



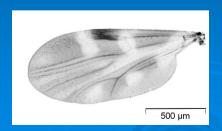


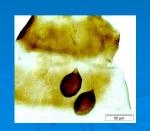




C. obsoletus







C. scoticus

## Female palpal measurements

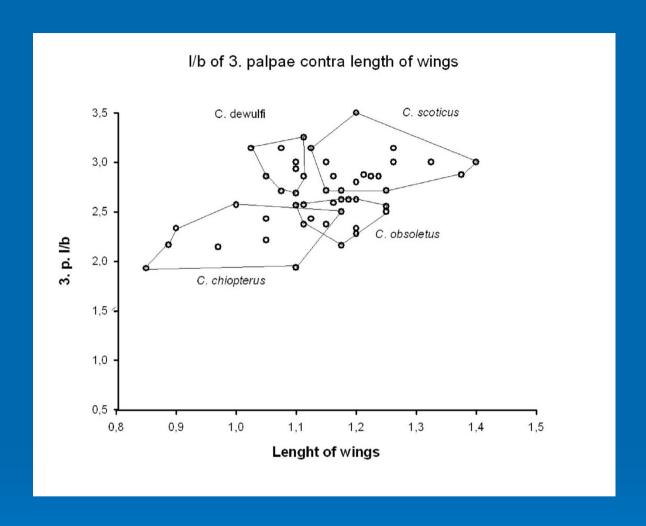
Species	N	2	3	4	5	Total	3 <sup>rd</sup> 1/w
C. obsoletus	20	58	51	24	29	161.9±9.9 <sup>A</sup>	2.4±0.4 <sup>A</sup>
C. scoticus	20	61	53	26	31	168.4±12,3 <sup>A</sup>	3.0±0.2 <sup>B</sup>
C. dewulfi	10	58	54	30	29	171.3±5.6 <sup>A</sup>	3.0±0.2 <sup>B</sup>
C. chiopterus	10	46	40	22	25	133.1±13.2 <sup>B</sup>	2.3±0.2 <sup>A</sup>

3.1/b length of third maxillary segment to the width. Means with the same letters are not significant different (Multiple comparison of Kruskal-Wallis).

### Female measurements

Species	N	Antenne	AR	Wing	Spermathek		ek
		(lenght)		(lenght)	1	2	Ratio 1/2
C. obsoletus	20	543±24 <sup>A</sup>	1.15±0.05 <sup>B</sup>	1.19±0.05 <sup>A</sup>	52	49	1.06
C. scoticus	20	541±29 <sup>A</sup>	1.21±0.03 <sup>C</sup>	1.24±0.08 <sup>C</sup>	60	57	1.05
C. dewulfi	10	475±20 <sup>B</sup>	1.16±0.03 <sup>BC</sup>	1.09±0.03 <sup>B</sup>	52	43	1.21
C. chiopterus	10	423±33 <sup>B</sup>	1.06±0.04 <sup>A</sup>	1.01±0.11 <sup>B</sup>	49	44	1.11

Antennal ratio (AR) of the female is the ratio of the sum of the lengths of the apical five segments of the the flagellum (11-15) to the sum of basal eight. Means with the same letters are not significant different (Multiple comparison of Kruskal-Wallis).



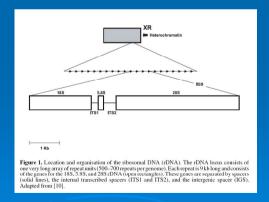
There is none or a very little overlap between the size to 3. 1/b relationships between the four species.

# Danish Culicoides species identified by molecular methods



# The molecular marker used is ITS1 (Ribosomal Internal Transcribed Region I).

Method adapted and basic reference sequences from Perrin et *al* 2006 (Montpellier-Strasbourg).



Cêtre-Sossah et al. 2004. Veterinary Research 35: 325-337.

Perrin et al. 2006. Medical and Veterinary Entomology 20: 219-228.



### Molecular test

- > 158 Culicoides specimens in ethanol.
- From obsoletus or pulicaris group.
- Morfological determination by S. A. Nielsen.
- > DNA purification by DNAzol.
- > PCR + sequence of ITS1.
- Sequence analysis (CLCbio; www.clcbio.com)



### Molecular results

Culicoides	N
- obsoletus	52
- scoticus	60
- dewulfi	10
- chiopterus	9
- pulicaris	10
- punctatus	10

- > 151 *Culicoides* specimens identified.
- 100% agreement between morphology and molecular data in both male and female Culicoides.
- Other species identified in Danish samples by molecular methods: circumscriptus, duddingstoni, festivipennis, newsteadi ..... more to come.

### C. obsoletus s.s.

- Intraspecies difference is <1%; in obsoletus s.s. polymorfism is found at 3 of 350 bases (N=52)</p>
- > Four types identified.
  - obs A
  - obs B
  - obs C
  - obs D
- All found as homo- and heterozygotes.

		20 I	40 6	0
France	GTAGGTGAACCTGCGGAAG		AAACATATAATGATGTTTCTTA	A 60
obs_A				. 22
obs_C				. 17
obs_B			• • • • • • • • • • • • • • • • • • • •	. 17
obs_D				. 22
Consensus		80	AAACATATAATGATGTTTCTTA	-
		80 I	100 12 I	
France	AAAAGGTATATGGTTGTC	GTTGTTATTGCAGCAGCTTT	GCCTTCACGGGCATTGCAGCTC	
obs_A				. 82
obs_C obs_B				. 77 . 77
obs_B				. 82
Consensus	AAAAGGTATATGGTTGTC		GCCTTCACGGGCATTGCAGCTC	
Conconcac	AAAAGGTATATGGTTGTG	140	160 18	-
-		T		
France obs A	GCTATAAAGATGTAGTTT		TGTGATTCAACAAAAAATTTCT	T 180
obs_A				. 137
obs_B				. 137
obs_D				. 142
Consensus	GCTATAAAGATGTAGTTT	ATTATGTCTTAAGCGGGTGC	TGTGATTCAACAAAAAATTTCT	Γ
		200	220 24	0
France	TGGGTAGCTTTATTGAAG	AGCTTTAAAGACTTGTTTGC	CCAAGGCCCCCTTAAAACTAG	240
obs_A				. 202
obs_C				. 197
obs_B				. 197
obs_D	TGGGTAGCTTTATTGAAG			. 202
Consensus	IGGGTAGCTTTATTGAAGA	260	CCAAGGCCCCCTTAAAACTAG1	
		T	-T	ĺ.
			GCATTGATTTTTTATCAAGATA	
obs_A				. 262
obs_C obs_B	A			257
obs_B	Α			. 262
Consensus	GATTCAGTTCGCTAGTATC	GCATATTCTTAACTGAGTAT	GCATTGATTTTTTATCAAGATA	
		320	340 36	0
France	CTTTGGAGTGAGTACAAA	 TGTACATGAAAATAAAATAA	AAAAAGATCAAAAACCTTAAAC	360
obs A				
obs_C				317
obs_B	C .			. 317
obs_D				. 322
Consensus	CTTTGGAGTGAGTACAAA		AAAAAGATCAAAAACCTTAAAC	3
		380 I		
France		TGGGTCGATGAAGACCGCA		
obs_A			349	
obs_C			344 340	
obs_B obs_D			340 337	
Consensus	GGGGGATCACTTGGCTCA		,,,,	
Consensus	COCCOMICACIIGGCICA	. 000 . 004		

### C. scoticus

- ▶ Intraspecies difference is ~3%; in scoticus polymorfism is found at 17 positions (N=60).
- > Five types identified
  - scot A1 and A2 (<1%)</li>
  - scot B1 and B2 (<1%)</li>
  - scot C
- All found as homo-and heterozygotes.

	20 40 60
scot A1	«AAACATATAATGATGTTTCTTT 22
scot A2	
	GTAGGTGAACCTGCGGAAGGATCATTATTGTATATTAA60
	<b>«</b>
scot_B2	<b>«</b>
scot_C	
Consensus	AAACATATAATGATGTTTCTTT
	80 100 120 I I
scot A1	
scot_A2	
France_scot	
scot_B1	
scot_B2	
scot_C	
Consensus	AAAAGGTATATATGGTTGTCGTTGTTATTGCAGCAGCTTTGCCTTTGCGGGCATTGCAGC
	T T
scot_A1	TCGCTCCAAAGATGTAGTTTATTGTCTTAAGCGGGTGCTGTAATTCAACAAAAAA-TT 141
scot_A2	
France_scot	
scot_B1	AT
scot_B2 scot_C	AT
	TCGCTNNAAAGATGTAGTTTATTATGTCTTAAGCGGGTGCTGTAATTCAACAAAAAA-TT
Consensus	200 220 240
	T T
scot_A1	
scot_A2	
France_scot	
scot_B1 scot_B2	
scot C	
Consensus	TCTTGGGTAGCTTTATTGAAGAGCTTTAAAGACTTGTTTGCCCAAGGCCCCCTTAAAACT
Conscisus	250 280 300
	TT
	AGTGATTCAATTCGCTAGTATGCATATTCCTTTATTGGGGTATGCATTGATTTATATCAA 261
scot_A2 France scot	
scot B1	
scot B2	261
scot_C	<b>G</b>
Consensus	AGTGATTCAATTCGCTAGTATGCATATTCCTTTATTGGGGTATGCATTGATTTATATCAA
	320 340 360
scot A1	GATACTTTGGAGTGAGTACGAATGTACATTTAAAAAATAAAAAAAA
scot_A1	320
France scot	C A T A 359
scot_B1	
scot_B2	
scot_C	
Consensus	
	380 400
scot A1	TAAACGGGGGATCCCTTGGCTCA-GGGTCGATGAA 354
scot A2	351
France_scot	
scot_B1	GACCG 355
scot_B2	GACCG 359
scot_C	338
Consensus	TAAACGGGGGATCACTTGGCTCATGGGTCGATGAAGACCG

## C. obsoletus s.s. - C. scoticus



	20 40 60	
scot A1	«AAACATATAATGATGTTTCTTTAAAAGGTATATATGGTTGTCGTTGTTATTGCAGCAGCT	60
scot_A1		55
scot C<		57
obs A	A	58
Consensus	AAACATATAATGATGTTTCTTTAAAAGGTATATATGGTTGTCGTTGTTATTGCAGCAGCT	
	80 100 120	
	1 1	400
scot_A1 scot_B1	TTGCCTTTGCGGGCATTGCAGCTCGCTCCAAAGATGTAGTTTATTATGTCTTAAGCGGGT	120
scot_B1		117
obs A	CAAT	118
Consensus	TTGCCTTTNCGGGCATTGCAGCTCGCTATAAAGATGTAGTTTATTATGTCTTAAGCGGGT	110
Consensus	140 160 180	
	T T	
scot_A1		
scot_B1		175
scot_C	<u>G</u>	176
obs_A	G	177
Consensus	GCTGTNATTCAACAAAAA - TTTCTTGGGTAGCTTTATTGAAGAGCTTTAAAGACTTGTT	
	200 220 240 I I I	
scot_A1	TGCCCAAGGCCCCTTAAAACTAGTGATTCAATTCGCTAGTATGCATATTCCTTTATTGG	239
scot_B1		235
scot_C		
obs_A		235
Consensus	TGCCCAAGGCCCCTTAAAACTAGTGATTCANTTCGCTAGTATGCATATTCCTTTATTGG	
	260 280 300	
scot A1		298
scot_B1	C	294
scot_C	A	295
obs_A	AAGAT.A	295
Consensus	GGTATGCATTGATTTAT-ATCAAGATACTTTGGAGTGAGTACNAATGTACATTTAAAAAT	
	320 340 360	
scot A1	AAAAAAAAAAAGATCAAAAAACCTTAAACGGGGGATCCCTTGGCTCA-GGGTCGATGAA	354
scot_A1		
scot C	ТТ.	338
obs_A	T	349
Consensus	AAAAAAAAAAGATCAAAAACCTTAAACGGGGGATCACTTGGCTCATGGGTCGATGAA	
	254	
	354 CG 355	
	- 338	
	349	
Consensus		
Consensus		

- Morphology and molecular data agree on 60 scoticus (male and female).
- Morphology and molecular data agree on 52 obsoletus s.s. (male and female).
- Difference between obsoletus s.s. and scoticus is 5-7%