



Minutes of the MedReoNet taxonomy meeting Strasbourg 16-20 March 2009

MedReoNet Workpackage 2: Regional Surveillance for vectors

Deliverable 2.2: Guide to the identification of *Culicoides* vectors and potential vectors present in the Mediterranean (European) basin, including molecular methods

The following people attended this session. Those in italics gave presentations:

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General objective:

To develop an identification tool to facilitate the morphological identification of Palearctic *Culicoides* species

Specific objectives:

To define consensually the taxonomic units (groups, complexes, and sibling species);

To present the first draft of the identification key (currently on French fauna);

To discuss and validate the diagnostic characters for the main Palearctic species;

The afternoon of the third day will focus on organization of standardized multicentric insecticide tests.



Chairmen: Miguel Miranda and Thomas Balenghien

Tuesday 17th March

Official opening and welcome (Professors Candolfi and Lude)

After the welcome introduction, Professor Candolfi (head of IPPTS) reminded the long history of *Culicoides* at the Medicine Faculty of Strasbourg. Professors Callot and Kremer gave years of work on *Culicoides*, especially on systematic and taxonomy, leaving us a huge and beautiful collection, useful for future taxonomy studies.

Currently, entomological activities on *Culicoides* are revived at the Faculty of Medicine with the come back of Jean-Claude Delécolle and the start of Bruno Mathieu's Phd on the systematic and taxonomy of the subgenus *Avaritia*.

To date, it is probably the first international meeting of people working on *Culicoides* taxonomy, (which is a difficult task). This meeting will produce fruitful exchanges to improve our understanding of systematic and to discuss on morphological and molecular tools.

After these words, Professor Lude (Dean of the Faculty of Medicine of Strasbourg) thanked the steering committee for the organization of this meeting in the Forum building of the Faculty of Medicine, and wished fruitful discussions.

Introduction and objectives

M. Miranda, leader of WP2

Miranda, leader of the WP2, thanked the steering committee for the organization. Then, he reminded the objectives of the WP2 of MedReoNet project, and especially the objective of the deliverable D22 "Guide to the identification of *Culicoides* vectors and potential vectors present in the Mediterranean basin, including molecular methods".

This deliverable is subdivided in two parts. On one hand, Cirad is the coordinator of an ongoing ring trial to assess the reliability of molecular tools currently used for the identification of *Culicoides* species. On the other hand, a key for the morphological identification of *Culicoides* adults, which would be available as a freeware, is currently developed under a collaboration between IPPTS (University of Strasbourg) and Cirad, with the method used by Francis Schaffner (UZH) for the identification key of European mosquitoes.

Thus, this meeting on *Culicoides* taxonomy was organized, in Strasbourg, with Jean-Claude Delécolle and Rudy Meiswinkel as experts, to help in the development of this identification tools by:

- defining consensually the taxonomic units (group, complex, and sibling species);
- presenting the preliminary version of the key;
- discussing and validating the diagnostic characters used in the key.

Then, it was underlined the necessity to include a list of species of the European Palearctic subregion in the final report of MedReoNet, because of the lack of published fauna for most of countries and the importance to add in the identification key information on species



ecology. The importance of a two-level key (one for non-specialists as surveillance actors and one for taxonomists) was reminded.

Moreover, apart from the taxonomy meeting, the organisation of a multi-centric assay to assess the *Culicoides* susceptibility to insecticides was discussed.

The Imicola Complex: taxonomy, ecology and biogeography

R. Meiswinkel

Culicoides taxonomy remains an unfinished business. For example, probably 40% of South-African species are not described, and the number of known species passed from 22 to 112 after Rudy's work. The subgenus *Avaritia* encompasses about 20 species clustered in groups and complexes. The Imicola Complex contains 6 African species: *C. imicola*, *C. kwagga*, *C. bolitinos*, *C. loxodontis*, *C. tuttifrutti*, and *C. miombo*.

Species classification is important because species from a same monophyletic group or complex may exhibit different bio-ecology such as trophic behaviours or breeding sites (moist soil exposed on sun for *C. imicola*, buffalo (or cattle) dungs for *C. bolitinos*, strictly elephant dungs for *C. loxodontis*, zebra dungs for *C. kwagga*, or rotting fruits (wild or cultivated) for *C. tuttifrutti*).

More generally, species classification can be difficult because of intra-specific morphological variations, which could or not reflect speciation event. Molecular tools could then be useful. Moreover, clear meaning of complex and group are still lacking for *Culicoides*, and some species are misclassified as *C. dewulfi* in the Obsoletus group or *C. griseascens* in the Pulicaris group. Numerous problems remain: allopatric species *C. bolitinos* and *C. brevitarsis* are virtually impossible to distinguish morphologically; European *C. montanus* may be a different species than the type from Central Asia; the taxonomic unit "subgroup", as for the Pseudopallidipennis subgroup, is probably a wrong term; it is impossible to distinguish morphologically *C. imicola* and *C. nudipalpis* (the latter species is found at the east of the Wallace line); it is not clear whether *C. pulicaris* and *C. lupicaris* are different species. Finally, in China, a huge work on *Culicoides* taxonomy is done and published, but without clear illustration, and with probably a lot of misidentification.

Conclusion (M. Miranda): it seems essential, for the morphological key under development, to add information on species ecology and to avoid subjective characters, and it could be useful to recommend a published iconography as reference.

Systematic and taxonomy of the genus *Culicoides*, what is coming next? (Claire Garros)

C. Garros, B. Mathieu, T. Balenghien, J.C. Delécolle

Claire Garros is an entomologist who worked for 6 years on systematic and taxonomy of *Anopheles*. She has been hired at Cirad recently and her work moves towards *Culicoides*.

Based on literature and discussions with *Culicoides* taxonomists, four main problems are identified in *Culicoides* systematics: phylogenetic relationships between and within subgenera are unknown; lack of uniformity in taxonomic categories; various species-groups not yet investigated on a worldwide basis; and the entire classification has not been published



in this entirety. Molecular tools are useful to complete some lacks but they have to be used in complement of the morphology.

Indeed, some subgenera are not monophylogenetic and within some subgenera there is no clear definition of taxonomic units such as group. Then, clear meaning for group, complex and sibling species were proposed.

Moreover, numerous morphological variations were described and it is difficult to know if they are intra or inter-specific variations. To answer these questions, reference collections should be developed including both voucher specimens and sequences (cytochrome B to follow the barcoding project).

Conclusion: it is needed to review subgenera phylogeny, to develop molecular tools and morphological key, and to use more molecular markers from large populations to clarify *Culicoides* taxonomy.

Culicoides diversity in Belgium and some taxonomic problems

Deblauwe I., De Deken G., Madder M., De Deken R., Fassotte C., Cors R., Losson B., Paternostre J., Haubruge E. & Simonon G.

From 20 OVI traps, 1 CDC trap, 2 suction traps operated routinely in Belgium, 45 species were identified, among these 84% belonged to the *Avaritia* subgenera, 10% to *Culicoides*, and 60% to the sibling species *C. obsoletus/scoticus*.

Among these specimens, a high level of atypical form (about 5%) was recorded:

- intermediary forms between *C. circumscriptus* and *C. salinarius*, with atypical forms on wing pattern and cibarium ornamentation;
- intermediary and atypical forms in the Pulicaris group, between *C. pulicaris* and *C. punctatus*, and *C. pulicaris* and *C. lupicaris*, and numerous variations in *C. newsteadi*. With molecular markers (microarray development on DNA ITS1 region), intermediary forms could cross with *C. lupicaris*, *C. pulicaris*. Moreover, morphological variations on *C. deltus* could be associated with the same ITS1 sequence;
- atypical forms of *C. brunnicans* and *C. duddingstoni*.

Conclusion: molecular analyses of these intermediary forms are required.

Culicoides species from the subgenus Culicoides in Catalonia (NE Spain)

Pagès, N., Muñoz-Muñoz, F., Talavera, S., Sarto, V., Lorca, C. and Nuñez, J.I.

Culicoides identification could be based on morphology (metric traits, non metric traits, wing pattern) and molecular assays. Difficulties exist in both morphology (intra-specific variations) and molecular tools (few available sequence, lack of morpho-molecular comparison, few new PCR tools).

In Catalonia, 6 species from the subgenus *Culicoides* were identified from 10 traps: *C. punctatus*, *C. pulicaris/lupicaris*, *C. fagineus*, *C. newsteadi* and *C. flavipulicaris*. Among these, 118 were sequenced for COI gene. At least 11 species were identified from phylogenetic trees: 2 cryptic species morphologically identified as *C. pulicaris*, 3 as *C. newsteadi*, and 2 as *C. fagineus*, while the 4 remaining species were identified as *C. lupicaris*, *C. subfagineus*, *C. flavipulicaris* and *C. punctatus*. Principal component analyses of morphological metric data was useful to distinguish species within the three groups of



species having similar wing pattern. Then, some allele-specific PCR were developed to identify the 11 species.

Danish *Culicoides* species of the *Obsoletus* group identified by morphological methods S. Nielsen and M. Kristensen

A method was presented to morphologically separate *C. obsoletus*, *C. scoticus*, *C. dewulfi* and *C. chiopterus*. From field-collected individuals, hypopygium and wing for male and head, wing, and spermatheca for female were observed and species identification was obtained using DNA assay.

Differences between *C. obsoletus* and *C. scoticus* were found using the ratio of the length and width of the third segment of the palpus, the antennal ratio (sum of the lengths of the apical five segments of the the flagellum (11-15) to the sum of basal eight) and the wing length. Finally, there were few overlaps crossing the palpus ratio and the wing length.

PCR identification showed a 100% agreement between morphology and molecular identification. ITS1 sequences were slightly variable within a species (less than 1% in *C. obsoletus* and about 3% in *C. scoticus*), when differences between *C. obsoletus* and *C. scoticus* were about 5 to 7%.

Conclusion: the importance of crossing different characters is highlighted to identify species.

Culicoides Internet Ressources: Future Directions S. Carpenter

Generally, there is a trade-off between accessibility, lifespan, funding and scientific details of web resources.

Web information on *Culicoides* is very limited. *Culicoides* are not included in projects such as Tree of life web project or Encyclopedia of life. The Ceratopogonid web page was created for specialists and, for example, it is not referenced on google. By googling *Culicoides*, IAH website appears after the wikipedia page, which is clearly unreliable. Using French as language, the two first responses of Google refer to the Cirad website on bluetongue and *Culicoides*.

IAH is developing a website (www.culicoides.net), which allows the accessibility to species pages created by John Boorman (about 350 species), and which is subdivided into following sections: general information, taxonomy, ecology and surveillance and molecular biology. The taxonomy section will allow e-learning (especially videos), species pages, identification key and literature. In the next future, connectivity to other websites will be developed and the John Boorman's data sheets will be updated. More generally, this kind of website could be a useful tool to develop teleconferencing, to disseminate results, and to give available full insect 3-D imaging. Evaluation of this website should be based on degree of "customer" satisfaction.



Traditional identification key
J.C. Delécolle et B. Mathieu

First, species could be divided into groups based on similar wing pattern, and then some morphological character could be used to identify specimens down species. French species could be split in 3 groups: wing without spot, with one dark spot, and the remaining species. Within the subgenus *Avaritia*, *C. chiopterus* could be identified by the eye pubescence, *C. dewulfi* by the unequal size of spermatheca, and *C. obsoletus* and *C. scoticus* could be easily differentiated as male. Moreover, *C. imicola* could show great variations in the wing pattern. Within the subgenus *Monoculicoides*, the main identification problem remains between *C. puncticollis* and *C. riethi*. Obviously, species with wing entirely clear are the most difficult to identify. Within the subgenus *Culicoides*, great variations are known within the Pulicaris group.

Conclusion: this classical dichotomic key could be considered as “has been”. In a near future, collaboration between taxonomists, computer specialists, and geneticists will allow to glimpse new tools more successful and more accurate for the identification and the classification of all *Culicoides* native from Europe or elsewhere

Presentation of the PhD plan of Bruno Mathieu

Briefly, Bruno Mathieu presents the plan of his starting PhD entitled “systematic and taxonomic revision of the subgenus *Avaritia* with a special interest for the *Obsoletus* group”. The aim is to resolve phylogenetic relationships between groups and within *Obsoletus* group, to develop morphological and molecular tools for *Culicoides* identification, and to map the species distribution. This thesis will provide a scheme for internal classification for the subgenus *Avaritia* and tools for surveillance activities and academic research.

Photographic and identification key to adult *Culicoides* species of the Palearctic region
Mathieu B., Chavernac D. & Delécolle J.C.

The objective is to develop a comprehensive morphological key to identify males and females in the aim to have a useful tool for surveillance activities and research. Thus, the key will be multi-access and available as a freeware. For each species, an identity card will give details on taxonomy, ecology, distribution and role as a vector.

Currently 20 wing characters were defined with 2 to 5 states of character for presence, size, position, or colour. Description was made using types from collections of Callot and Kremer, and Delécolle. Characters were encoded into a database.

To date, 85 species are encoded for 20 characters on wing pattern leading to 65 states of characters described by 250 pictures. In the future, more species and other characters (from microscopy) will be included, picture database completed and the freeware delivered online.

Bruno gave a demonstration of the key. Some recommendations were made:

- avoid terms as slightly or mostly;
- show pictures for each choice in the same screen to be able to compare;
- add an optimization process;
- add the possibility to upload a personal picture library.



Wednesday 18th March

Resume of Tuesday talks (Thomas Balenghien)

This talk gives the opportunity to discuss about taxonomy units. It was highlighted the usefulness of the complex as a taxonomic unit. Finally, it was agreed that complex could group species for which males could be easily identified. In the 2008 EFSA report, it was advised to use Obsoletus group for *C. obsoletus*, *C. scoticus* and *C. montanus*. The term Obsoletus group should not be used instead of *Avaritia*. Moreover, it was recommended that in each published paper, the material and methods section should clearly described the identification method.

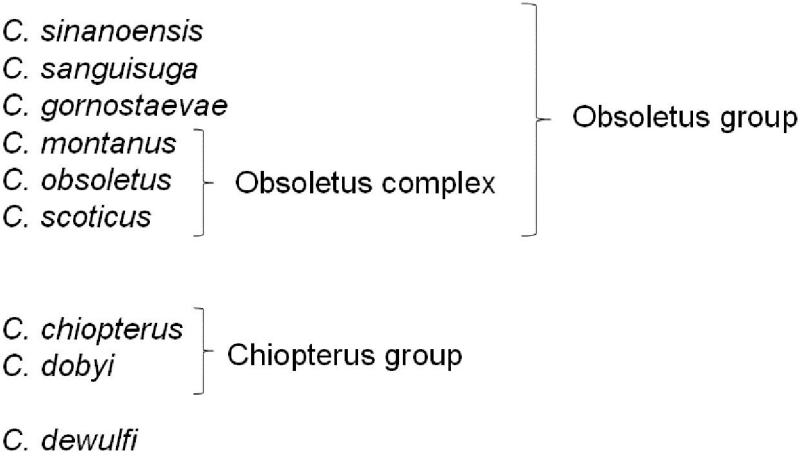
In conclusion, the following definitions were consensually adopted and must use in future papers:

Species group refers to a grouping of phylogenetically closely related species. Female diagnose is possible on wing pattern for example.

Species complex refers to a grouping of phylogenetically closely related species for which it is difficult or impossible to differentiate them as male or as female. The species belonging to a complex are called sibling species or cryptic species.

Informal group names are treated as vernacular names. Names are printed in roman type with the first letter capitalized even though the name of a nominal species is used in the combination. The oldest species gives its name to the group or complex species.

Example of definition application to the Palearctic species of the *Avaritia* subgenus:



Round table discussion on morphological characters to be included in the identification key



Tuesday 19th March

Round table discussion on slides from UK for species identification

Announcement of the 1st International meeting on *Culicoides* taxonomy

Bruno Mathieu, Jean-Claude Delécolle, and Ermanno Candolfi were glad to announce the organisation of the first international meeting on *Culicoides* taxonomy in Strasbourg in April 2010.

WHO test applied on *Culicoides*: interest and expected results of multicentric study

R. Venail, B. Mathieu, M.L. Setier-Rio, T. Balenghien

Within the framework of MedReoNet, a multicentric assay to assess the sensibility of *Culicoides* to insecticide using WHO tubes. In complement of the exposed protocol, it was decided to maintain collected specimens during 24 hours (to avoid rapid high level of mortality), and then to perform the test placing about 50 unsorted individuals by tube. Videos of each protocol step will be provided to standardize the procedures.

Persons interested are: Roger Vénail and Marie Laure Setier-Rio (EID-Med as leader with Cirad), Simon Carpenter (IAH), Gil De Deken (ITM), Isabel Fonseca + José Meireles (FMV), Maria Goffredo (IZS to be confirmed), Karien Labuschagne (OVI), Xavier Lucientes (UNIZAR), Miguel Miranda (UIB), Nonito Pages (CRESA), Soufien Sghaier (IRVT), and Doreen Wener (DEI + FLI?).

EID-Med will provide to all a detailed protocol before June.

Conclusions (Miguel Miranda)

Miguel Miranda reminded the general and specific objectives and emphasized that some objectives had been already reached.

As general conclusions, he highlighted:

- the need of linking morphology and molecular taxonomy;
- the agreement about the definition of group and complex terms;
- the need of effective connectivity and reliable sources of information.

Finally, he recommended:

- morphological studies combined with DNA Barcoding analysis are proposed (C. Garros will be the coordinator);
- a list of the Western Europe *Culicoides* species will be developed (J.C. Delecolle, B. Mathieu, C. Garros and R. Meiswinkel will be the coordinators);
- recommendations to authors about terminology and mat. & met. details about identification of *Culicoides* species will be given (D. Werner will be the coordinator);



- when possible, sequencing and incorporation of the sequences to Genbank is recommended;
- multicenter studies covering large scale populations for Culicoides phylogeny are suggested to be conducted (N. Pages will be the coordinator);
- intraspecific polymorphisms measured by morphometrics and molecular methods are needed (i.e. Obsoletus complex).

Simon Carpenter underlined the importance to find other source of funding because of the future end of the MedReoNet project. Frederigo Santini answered that European programs as COST (European Cooperation in Science and Technology, <http://www.cost.esf.org/>) could be used after the end of MedReoNet.

At the end, Jean-Claude warmly thanked all participants for their coming to Strasbourg.