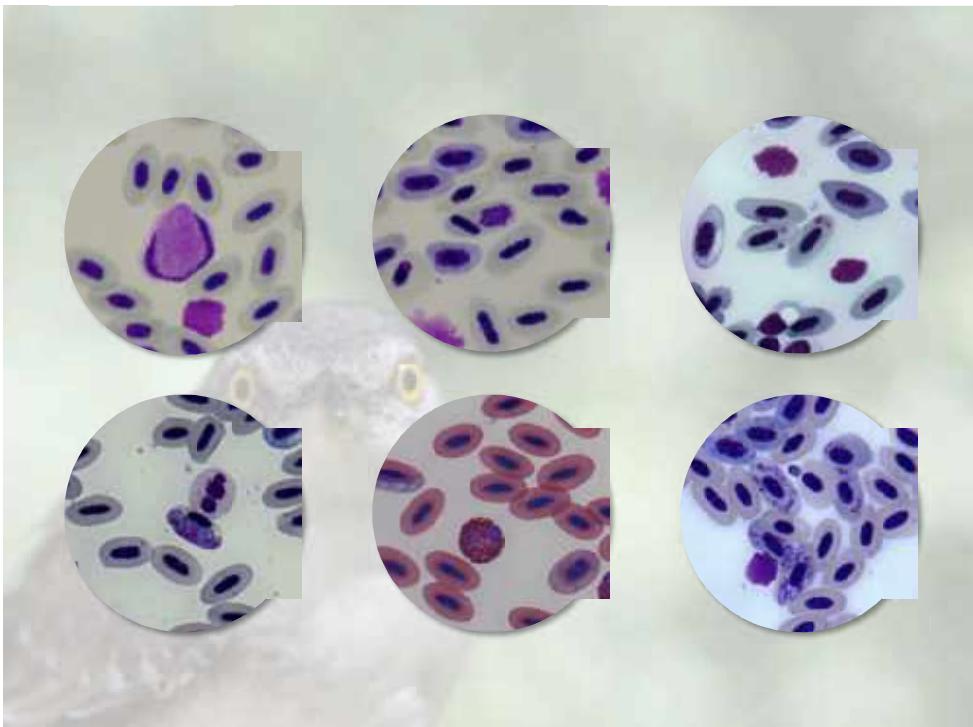


1st WIMANET Summer School

Mohelno, Czech Republic
September 2024



Funded by
the European Union

PROGRAM

| Sunday, September 1st | | |
|--|--|--|
| 15:30 | Departure from the Prague airport | |
| 18:30 | Estimated arrival time at the Mohelno field station | |
| Monday, September 2nd – Introduction, birds, Haemosporidians | | |
| 9:00 | Introduction (Theoretical) | Pavel, Jenny, Ravinder |
| 10:00 | Break, Students and teachers ice-break | Carolina |
| 11:30 | Haemosporidians: diversity, phylogeny, cophylogeny, hosts (Theoretical) | Ravinder |
| 13:30 | Lunch and shopping in Mohelno | |
| 16:00 | Bird catching, ringing, blood sampling, preparation of blood smears (Theoretical/Practical/Hands-on) | Dimitar and Ondra |
| 17:00 | Students' presentation | Vaidas, Carolina |
| 19:00 | Dinner and discussions | |
| Tuesday, September 3rd – Haematology and parasites | | |
| 7:00 | Trapping birds, slide preparation (Practical/Hands-on) | Ondra, Vaidas, Dimitar, others |
| 10:30 | Break | |
| 11:00 | Haematology: microscopy (Theoretical/Hands-on) | Ravinder (supporting teachers Carolina, Jenny, Vaidas) |
| 12:30 | Lunch | |
| 14:00 | Parasites: morphology, microscopy (Theoretical/Practical) | Vaidas and Carolina |
| 15:30 | Break | |
| 16:00 | Parasites: morphology, microscopy (Hands-on) | Vaidas, Carolina, Dimitar |
| 17:30 | Students projects | Vaidas, Carolina, Ravinder, Dimitar, Jenny |
| 18:30 | Vector catching – depends on the weather (Hands-on) | Bruno |
| 19:00 | Dinner and discussions | |
| Wednesday, September 4th – Vectors | | |
| 8:30 | Vector catching (Practical/Hands-on) | Bruno, Carolina |
| 9:00 | Vector diversity, life cycle, morphological and molecular (Theoretical/Practical) | Bruno |
| 10:30 | Break | |
| 11:00 | Sorting material and dissections (Practical/Hands-on) | Bruno, Carolina |
| 12:30 | Lunch | |
| 14:00 | Vector competence and salivary gland preparation (Practical/Hands-on) | Carolina |
| 15:30 | Break | |
| 16:00 | Vector dissection and salivary gland preparation (Hands-on) | Bruno, Carolina |
| 18:00 | Students Projects | Bruno, Carolina |
| 19:00 | Dinner and discussion | |
| Thursday, September 5th – Markers, genomics, databases | | |
| 9:00 | Vector or bird catching (Practical/Hands-on) | Flexible, Ondra, Vaidas, Bruno |
| 10:30 | Break | |
| 11:00 | Markers, genomics, transcriptomics (Theoretical) | Ricardo, Xi, Jenny |
| 12:30 | Lunch | |
| 14:00 | Sequencing, database, phylogeny | Ricardo, Xi, Jenny |
| 15:30 | Sequence data handling, database search, phylogeny | Ricardo, Xi, Jenny |
| 17:00 | Break | |
| 18:00 | Students Projects | Ricardo, Xi, Jenny |
| 19:00 | Dinner (Mohelno?) | |

| Friday, September 6th – Community | | |
|---|---|-----------------------------------|
| 9:00 | Communities, switches, cophylogeny (Theoretical) | Ricardo, Xi, Jenny |
| 10:30 | Break | |
| 11:00 | Communities, switches, cophylogeny (Practical) | Ricardo, Xi, Jenny |
| 12:30 | Lunch | |
| 14:00 | Students practice (microscopy and insect dissection) (Hands-on) | Carolina, Vaidas, Dimitar, others |
| 16:00 | Break | |
| 16:30 | Students projects, evaluation, summary | Vaidas, Carolina |
| 19:00 | Dinner (barbecue) and discussions | |
| Saturday, September 7th | | |
| 10:00 | Departure time from Mohelno field station | |
| 14:00 | Estimated time for arrival at Prague airport | |

RECOMMENDED READINGS**Books**

- Atkinson, C. T., Thomas, N. J. & Hunter, D. B. (eds.). (2008) *Parasitic diseases of wild birds*. Blackwell Publishing, Ames, Iowa.
- Campbell, T.W. & Ellis, C.K. (2013) *Avian and exotic animal hematology and cytology*. John Wiley & Sons.
- Clark, P., Boardman, W. & Raidal S. (2009) *Atlas of clinical avian hematology*. John Wiley & Sons, 2009.
- Coatney, G. R., Collins, W. E., Warren, M. & Contacos, P. G. (1971) *The primate malarias*. Washington: US Government Printing Office.
- Garnham, P. C. C. (1966) *Malaria parasites and other Haemosporidia*. Blackwell Scientific Publications, Oxford, U.K.
- Telford, S. R. (2009) *Hemoparasites of Reptilia: Color atlas and text*. CRC Press.
- Valkiūnas, G. (2005) *Avian malaria parasites and other haemosporidia*. CRC Press.
- Santiago-Alarcon, D. & Marzal, A. (eds.) (2020) *Avian malaria and related parasites in the tropics*. Ecology, Evolution and Systematics, Springer.

Chapters

- Atkinson, C. T. & van Riper III, C. (1991) Pathogenicity and epizootiology of avian hematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*. pp. 19-48, In: *Bird-Parasite Interactions, Ecology, Evolution and Behavior*, Loya, J. E. & Zuk, M. (editors). Oxford University Press, Oxford.
- Desser, S. S. & Bennett, G. F. (1993) The genera *Leucocytozoon*, *Haemoproteus* and *Hepatocystis*. In: Kreier, J. P. & Baker, J. R. (Eds.) *Parasitic Protozoa*. Second edition. Academic Press, 4, pp. 273-307.

Published articles

- Antinori, S., Bonazzetti, C., Giacomelli, A., Corbellino, M., Galli, M., Parravicini, C. & Ridolfo, A.L. (2021) Non-human primate and human malaria: Past, present and future. *Journal of Travel Medicine* 28, 1-14.
- Bensch, S., Canbäck, B., DeBarry, J.D., Johansson, T., Hellgren, O., Kissinger, J.C., Palinauskas, V., Videvall, E. & Valkiūnas, G. (2016) The genome of *Haemoproteus tartakovskyi* and its relationship to human malaria parasites. *Genome Biology and Evolution* 8, 1361-1373.
- Bensch, S., Hellgren, O. & Pérez-Tris, J. (2009), MalAvi: A public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Molecular Ecology Resources* 9, 1353- 1358.
- Bensch, S., Inumaru, M., Sato, Y., Lee Cruz, L., Cunningham, A. A., Goodman, S. J., Levin, I. I., Parker, P. G., Casanueva, P., Hernández, M., Moreno-Rueda, G. & Rojo, M. (2020) Contaminations contaminate common databases. *Molecular Ecology Resources*, 1755-0998.13272.

Böhme, U., Otto, T. D., Cotton, J. A., Steinbiss, S., Sanders, M., Oyola, S. O., Nicot, A., Gandon, S., Patra, K. P., Herd, C., Bushell, E., Modrzynska, K. K., Billker, O., Vinetz, J. M., Rivero, A., Newbold, C. I. & Berriman, M. (2018) Complete avian malaria parasite genomes reveal features associated with lineage-specific evolution in birds and mammals. *Genome Research* 28, 547-560.

Cox, F. E. G. (2010) History of the discovery of the malaria parasites and their vectors. *Parasites & Vectors* 3:5.

Ellis, V., Bensch, S., & Canbäck, B. (2017) MalaviR: an R interface to MalAvi. R package version 0.1. 0. GitHub. Retrieved from <https://github.com/vincenzoellis/malavir>.

Fecchio, A., Chagas, C. R., Bell, J. A. & Kirchgatter, K. (2020). Evolutionary ecology, taxonomy, and systematics of avian malaria and related parasites. *Acta Tropica*, 204, 105364.

García-Longoria, L., Ahrén, D., Berthomieu, A., Kalbskopf, V., Rivero, A. & Hellgren, O. (2023) Immune gene expression in the mosquito vector *Culex quinquefasciatus* during an avian malaria infection. *Molecular Ecology* 32, 904-919.

Hellgren, O., Križanauskienė, A., Valkiūnas, G. & Bensch, S. (2007) Diversity and phylogeny of mitochondrial cytochrome B lineages from six morphospecies of avian *Haemoproteus* (Haemosporida: Haemoproteidae). *Journal of Parasitology* 93, 889-896.

Martinsen, E. S., Perkins, S. L. & Schall, J. J. (2008), A three-genome phylogeny of malaria parasites (Plasmodium and closely related genera): Evolution of lifehistory traits and host switches. *Molecular Phylogenetics and Evolution* 47, 261-273.

Martinsen, E. S., McInerney, N., Brightman, H., Ferebee, K., Walsh, T., McShea, W. J., Forrester, T. D., Ware, L., Joyner, P. H., Perkins, S. L., Latch, E. K., Yabsley, M. J., Schall, J. J. & Fleischer, R. C. (2016) Hidden in plain sight : Cryptic and endemic malaria parasites in North American white-tailed deer (*Odocoileus virginianus*). *Science Advances* 2, e1501486.

Mathieu, B., Cêtre-Sossah, C., Garros, C., Chavernac, D., Balenghien, T., Carpenter, S., Setier-Rio, M.-L., Vignes-Lebbe, R., Ung, V., Candolfi, E. & Delécolle J.-C. (2012). Development and validation of IIKC: an interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the Western Palearctic region. *Parasites Vectors* 5, 137 (2012). <https://doi.org/10.1186/1756-3305-5-137>.

Pacheco, M.A. & Escalante, A.A. (2023) Origin and diversity of malaria parasites and other Haemosporida. *Trends in Parasitology*, 1-16.

Palinauskas, V., Valkiunas, G., Bolshakov, C. V. & Bensch S. (2008) *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. *Experimental Parasitology* 120:372-380.

Pérez-Tris, J., Hasselquist, D., Hellgren, O., Krizanauskienė, A., Waldenstrom, J. & Bensch, S. (2005) What are malaria parasites? *Trends in Parasitology* 21:209-211.

Pérez-Tris, J., Hellgren, O., Krizanauskienė, A., Waldenström, J., Seondi, J., Bonneaud, C., Fjeldsa, J., Hasselquist, D. & Bensch, S. (2007) Within-host speciation of malaria parasites. *PLoS One* 2:e234.

Perkins, S. L. (2014) Malaria's many mates: past, present, and future of the systematics of the order Haemosporida. *Journal of Parasitology* 100:11-25.

Ricklefs, R. E. & Outlaw, D. C. (2010) A molecular clock for malaria parasites. *Science* 329, 226-229.

1st WIMANET Summer School

Mohelno, CZ - 2024

- Rivero, A. & Gandon, S. (2018) Evolutionary Ecology of Avian Malaria: Past to Present. *Trends in Parasitology* 34, 712–726.
- Schall, J. J. 1996. Malarial parasites of lizards: diversity and ecology. *Advances in Parasitology* 37:255-333.
- Ursula, H., Rüdiger, K., Frank, M. & Monika, R. (2014) Blood parasites in reptiles imported to Germany. *Parasitology Research* 113, 4587–4599.
- Valkiūnas, G. & Iezhova, T. A. (2018) Keys to the Avian Malaria Parasites. *Malaria Journal*, 17, 212, doi:10.1186/s12936-018-2359-5.
- Valkiūnas, G. & Iezhova, T. A. (2022) Keys to the Avian *Haemoproteus* Parasites (Haemosporida, Haemoproteidae). *Malaria Journal*, 21, 269, doi:10.1186/s12936-022-04235-1.
- Valkiūnas, G. & Iezhova, T.A. (2023) Insights into the Biology of *Leucocytozoon* Species (Haemosporida, Leucocytozoidae): Why Is There Slow Research Progress on Agents of Leucocytozoonosis? *Microorganisms*, 11, 1251, doi:10.3390/microorganisms11051251.
- Valkiūnas, G., Iezhova, T. A., Kržanauskienė, A., Palinauskas, V., Sehgal, R. N. M. & Bensch, S. (2008) A comparative analysis of microscopy and PCR-based detection methods for blood parasites. *Journal of Parasitology* 94, 1395–1401.
- Videvall, E. (2019) Genomic advances in avian malaria research. *Trends in Parasitology* 35, 254–266.

STUDENTS PROJECTS

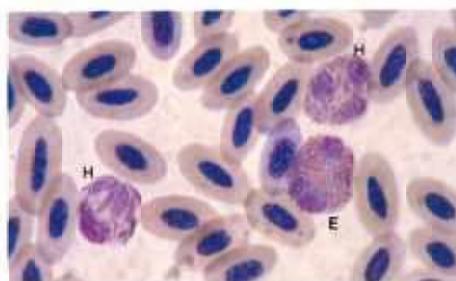
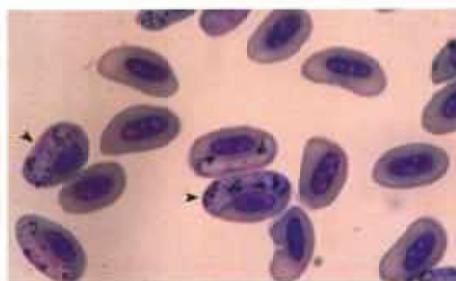
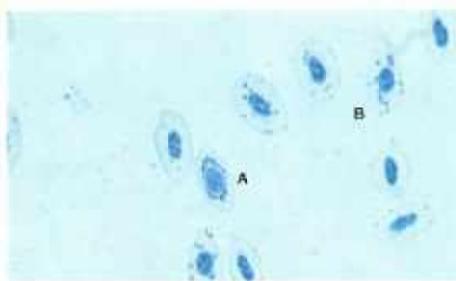
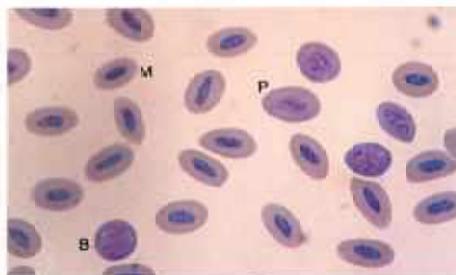
This project aims to encourage the students to put into practice what was taught in the theoretical, practical and hands-on lectures during this Summer School. There is no evaluation or grade related to this activity and the outcome will not influence the certificate given to all the attendees.

Instructions

- Students will be previously assigned to different groups
- Each group will draw lot a case study to work with in the next days.
- At each day, the students will have one hour in the program to work with their projects.
- We prepared a set of questions that the student should be able to answer by the end of this activity. It is not mandatory to answer all questions.
- On the last day, each group will present their results in a 5- to 10-minutes presentation.

Questions

1. Did you find any blood parasites? If so, what was the prevalence of infection in your study case?
2. How many birds have single infections? How many had co-infections?
3. What was the parasitemia in infected birds?
4. Which parasites (genus/subgenus) were found in your study case?
5. Did the differential WBC count differ from infected to uninfected individuals?
6. Which parasite lineages might be involved in the infections detected?
7. What other parasite lineages can be found in this bird species/region?
8. What are the expected vectors of these parasites? Based on information from caught insects in Mohelno, can there be potential vectors of the parasites found in your study case?
9. Which other bird species these parasites can infect?
10. Where on phylogenetic tree these parasites go?
11. Did you detect any co-infections from the sequence, and how did you identify each lineage?
12. Which lineages appear to be the most generalist one in this community?

Figure 9:

ERYTHROCYTE MORPHOLOGY (1000 X)

Stages of RBC development found in peripheral blood.

B - Basophilic erythroblast
P - Polychromatophilic erythrocyte
M - Mature erythrocyte

Figure 10:

RETICULOCYTES (NMB, 1000 X)

A - Reticulocyte with complete ring of reticulum around nucleus.
B - Reticulocyte with five or more clumps of chromatin.

Figure 11:

BLOOD PARASITE (1575 X)

Erythrocytes infected with Hemocystus sp. (arrows).

Figure 12:

HETEROPHILS / EOSINOPHIL (1575 X)

It is easy to distinguish these cells when seen together as in this figure. Differences in size, shape and color of granules are important features.

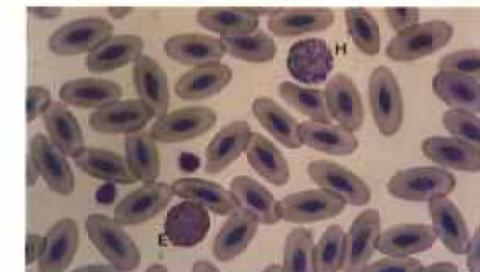
H - Heterophil
E - Eosinophil

Figure 13:

HETEROPHIL / EOSINOPHIL (1575 X)

Side by side view of a heterophil and an eosinophil. The heterophil has photographed with an artefactual blue hue, but the different shapes of the cell's granules are evident.

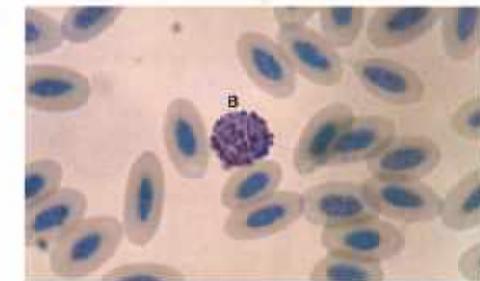
H - Heterophil
E - Eosinophil

Figure 14:

BASOPHIL (1575 X)

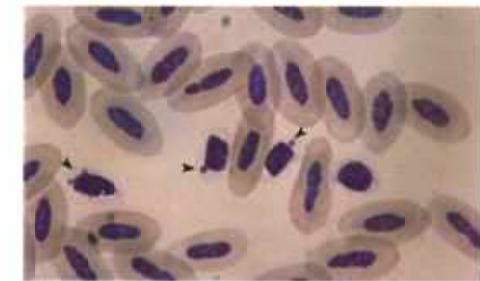
This cell is very similar to the mammalian basophil. Occasionally a degranulated basophil may be found.

B - Basophil

Figure 15:

THROMBOCYTES (1575 X)

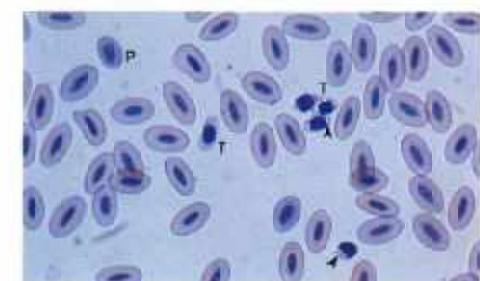
These pleomorphic cells often have 1 - 3 magenta granules in the cytoplasm (arrows).

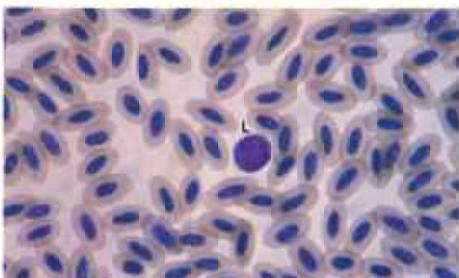
Figure 16:

THROMBOCYTES (1000 X)

Upon degranulation, thrombocyte granules become vacuoles (arrows).

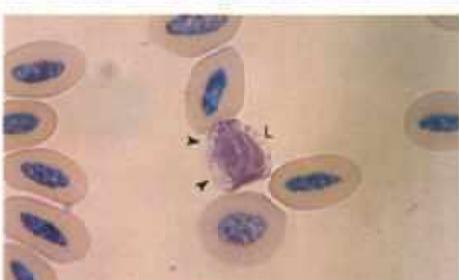
T - Thrombocyte
P - Polychromatophilic erythrocyte



Figure 17:

LYMPHOCYTE (1000 X)

Lymphocytes are variable in size. This is a small (I) lymphocyte.

Figure 18:

LYMPHOCYTE (1575 X)

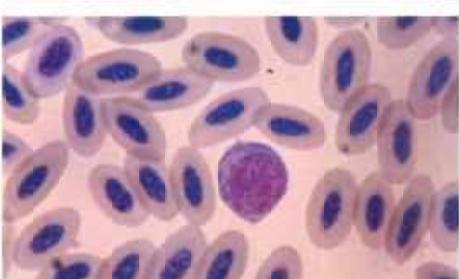
This is a large (II) lymphocyte. These cells may or may not have multiple magenta granules in the cytoplasm (arrows).

Figure 19:

LYMPHOCYTE / THROMBOCYTES (1000 X)

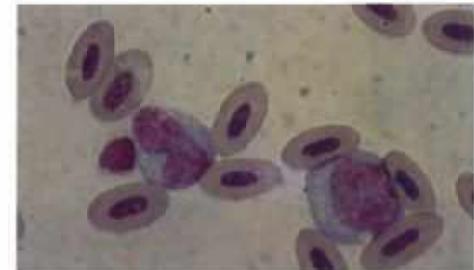
This figure compares four thrombocytes with one small (I) lymphocyte.

T - Thrombocyte
L - Lymphocyte

Figure 20:

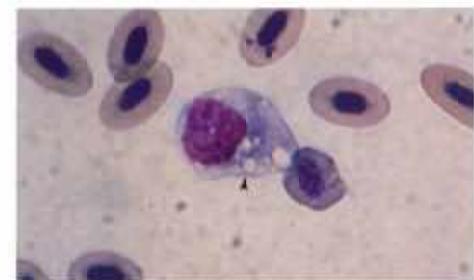
LYMPHOCYTE / MONOCYTE ? (1575 X)

It can be difficult to distinguish lymphocytes and monocytes. This cell, probably a lymphocyte, could be confused with a monocyte due to its size and shape.

Figure 21:

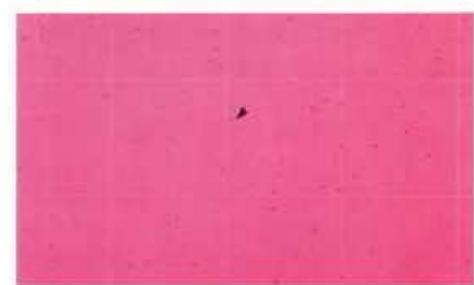
MONOCYTES (1575 X)

Monocytes are large cells with irregular borders and variably shaped nuclei.

Figure 22:

MONOCYTE (1575 X)

There are often vacuoles in the cytoplasm of monocytes (arrow).

Figure 23:

PHLOXINE STAIN (100 X)

This is an example of the appearance of cells counted with the Eosinophil Unocette®. All the orange - pink cells are granulocytes (arrow).

Figure 24:

NATT AND HERRICK'S SOLUTION (100 X)

This is a view of cells counted with Watt and Herrick's diluent. The darker staining cells are the leucocytes (arrow).

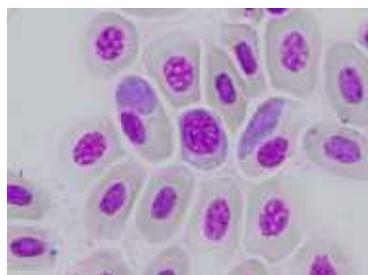
Other blood parasites - reptiles



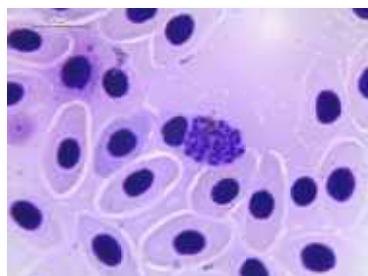
Hepatozoon sp. infecting an erythrocytes of a dark-headed false boa (*Pseudoboa neuwiedii*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



Hepatozoon sp. outside of erythrocytes in a Brazil's lancehead snake (*Bothrops brazili*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.

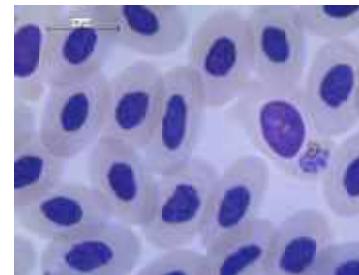


Non-pigmented Haemosporida (possibly *Garnia* sp.) infecting an erythrocytes of Turnip-tail gecko (*Thecadactylus rapicauda*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



Plasmodium (Sauramoeba) kentropyxi infecting an erythrocytes of whiptail lizard (*Cnemidophorus grammivagus*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.

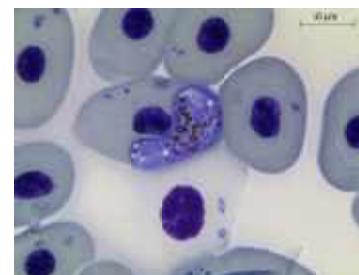
Other blood parasites - reptiles



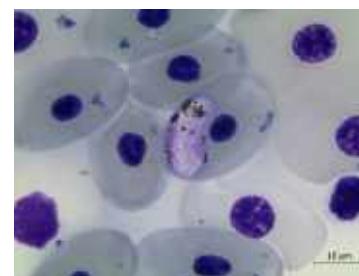
Plasmodium (Carinamoeba) carmelinoi infecting an erythrocytes of Amazon racerunner (*Ameiva ameiva*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



Hepatozoon simidi outside of erythrocytes in a Colombian wood turtle (*Rhinoclemmys melanosterna*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



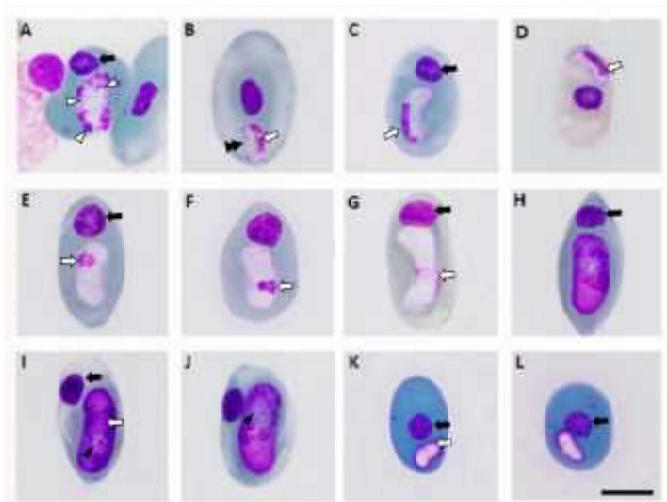
Hemocystidium sp. infecting an erythrocytes of the Savanna side-necked turtle (*Podocnemis vogli*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



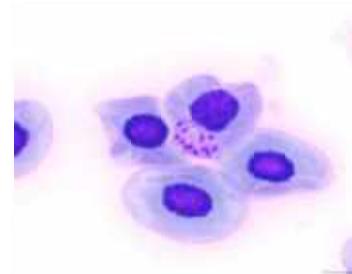
Hemocystidium sp. infecting an erythrocytes of the Savanna side-necked turtle (*Podocnemis vogli*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.

Other blood parasites - reptiles

Hepatozoon caimani outside of erythrocytes in the spectacled caiman (*Caiman crocodilus*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



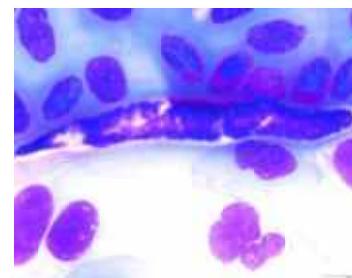
Hemogregarina sp. infecting erythrocytes of Savanna side-necked turtle (*Podocnemis vogli*) and Colombian wood turtle (*Rhinoclemmys melanosterna*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.

Other blood parasites - amphibians

Dactylosoma sp. infecting an erythrocytes of the marsh frog (*Pelophylax ridibundus*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



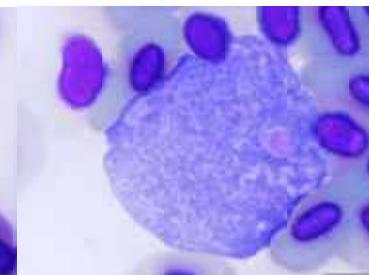
Lankesterella sp. infecting an erythrocytes of the marsh frog (*Pelophylax ridibundus*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



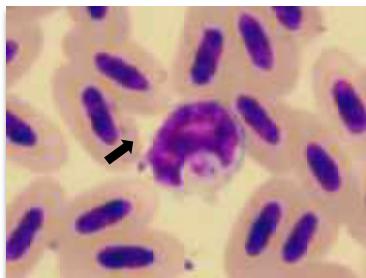
Microfilaria infecting a marsh frog (*Pelophylax ridibundus*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



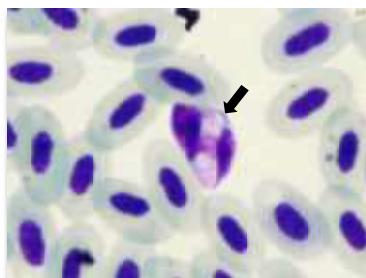
Trypanosoma sp. infecting a marsh frog (*Pelophylax ridibundus*). Methanol-fixed, Giemsa-stained. Source: G. Liberato.



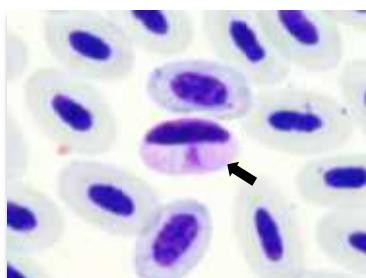
Other blood parasites - birds



Isospora sp., (=*Atoxoplasma* sp.) within a lymphocyte of a house sparrow (*Passer domesticus*). Methanol fixed, Giemsa stained.



Lankesterella bivacuolata, within a lymphocyte of a black bird (*Turdus merula*). Methanol fixed, Giemsa stained.

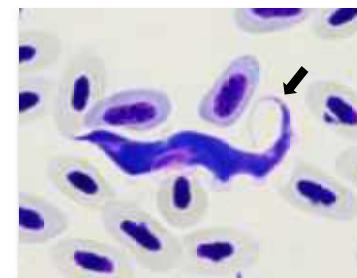


Lankesterella kabeeni, within a thrombocyte of a sedge warbler (*Acrocephalus schoenobaenus*). Methanol fixed, Giemsa stained.

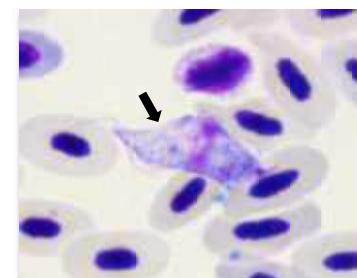


Microfilaria infecting a toco toucan (*Ramphastos toucan*). Rosenfeld stain.

Other blood parasites - birds



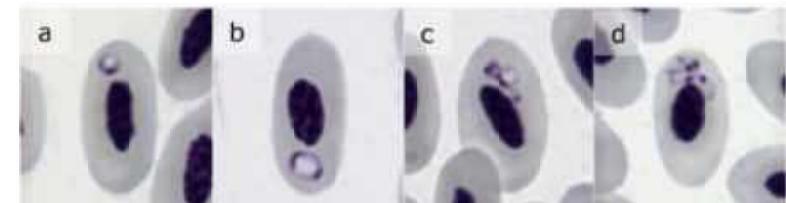
Trypanosoma avium infecting a blackbird (*Turdus merula*). Methanol-fixed, Giemsa-stained.



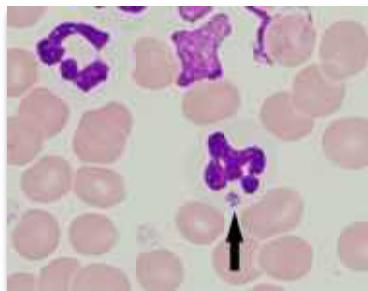
Trypanosoma everetti a blackbird (*Turdus merula*). Methanol-fixed, Giemsa-stained.



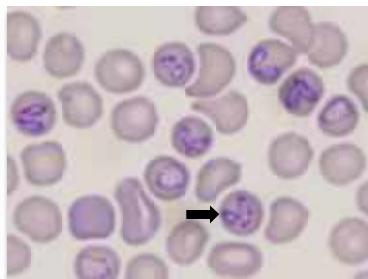
Hepatozoon albatrossi in the lymphocytes of a black-browed albatross (*Thalassarche melanophrys*). Source: [10.1016/j.vetpar.2016.12.001](https://doi.org/10.1016/j.vetpar.2016.12.001)



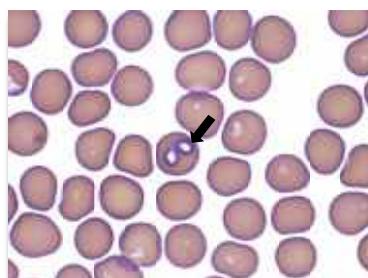
Babesia ugwidensis infecting erythrocytes of a in a Cape cormorant (*Phalacrocorax capensis*). Source: [10.1016/j.vetpar.2016.12.001](https://doi.org/10.1016/j.vetpar.2016.12.001)

Other blood parasites - mammals

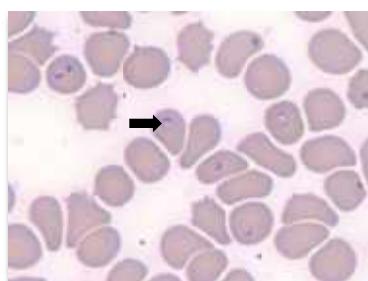
Ehrlichia ewingii within a neutrophil of domestic dog (*Canis lupus familiaris*). Romanovski stain. Image by NCVP



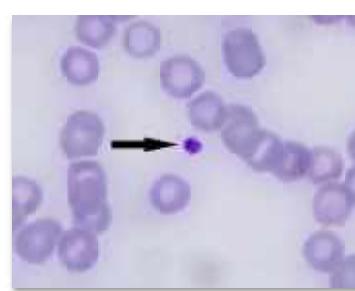
Babesia canis within an erythrocyte of domestic dog (*Canis lupus familiaris*). Romanovski stain. Image by NCVP.



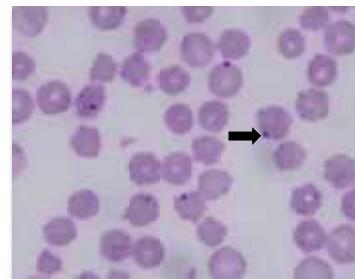
Babesia canis within an erythrocyte of domestic dog (*Canis lupus familiaris*). Giemsa stain.



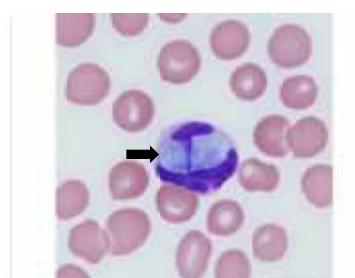
Cytauxzoon felis within an erythrocyte of a jaguar (*Panthera onca*). Romanovski stain.



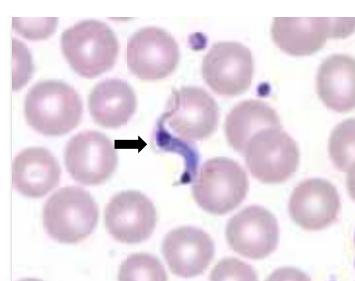
Anaplasma platys within an erythrocyte of a domestic dog (*Canis lupus familiaris*). Romanovski stain. Image by NCVP.



Anaplasma marginale within an erythrocyte of bovine (*Bos taurus*). Romanovski stain. Image by NCVP.

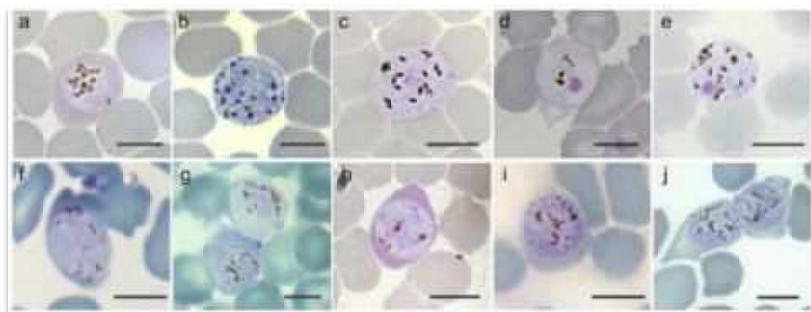


Hepatozoon canis within neutrophils of domestic dog (*Canis lupus familiaris*). Giemsa stain. doi: [10.1016/j.ijpara.2018.12.001](https://doi.org/10.1016/j.ijpara.2018.12.001)



Trypanosoma cruzi in human blood. Giemsa stain. Image by CDC.

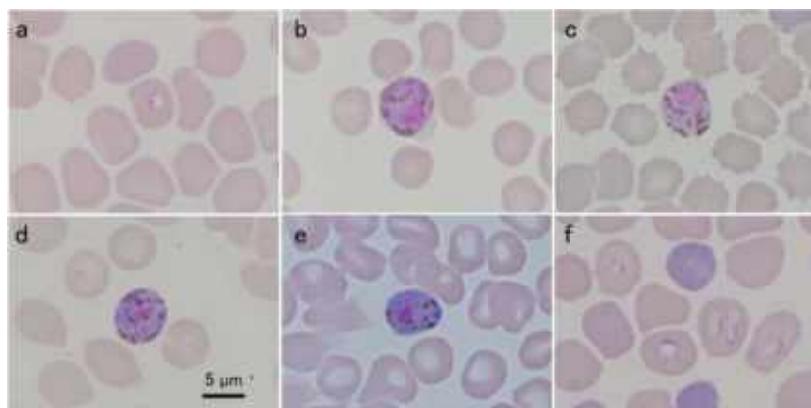
Other blood parasites - mammals



Nycteria spp. gametocytes within erythrocytes of two bat species:

Nycteris spp. (a-e) and *Rhinolophus* spp. (f-j). doi:

<https://doi.org/10.1016/j.ijpara.2015.01.008>.



Polychromophilus spp. within erythrocytes of *Miniopterus fuliginosus* (a-e) and *Myotis macrodactylus* (f) two bat species. Trophozoite (a), microgametocytes (b) and macrogametocytes (d, e) of *P. melanipherus*. Trophozoite of *P. murinus* (f). Giemsa stain. doi:

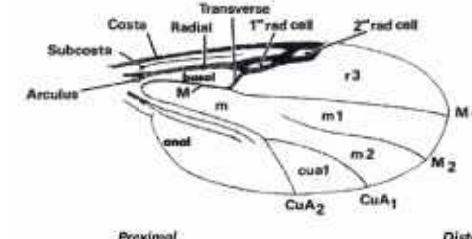
<http://dx.doi.org/10.1007/s00436-022-07592-7>.

Introduction to morphology and some taxonomic recommendations for a correct use of the IIKC *Culicoides* (Diptera: Ceratopogonidae)

Note: The IIKC Key is developed to identify *Culicoides* females from slide mounted specimens by observation on a microscope. We don't recommend the use on a stereomicroscope.

- Wings

General view of a *Culicoides* wing:



Note: Ideally, wings observation has to be done on a microscope with a low magnification (2,5x or 4x, not x10).

Capital letters are used to name the veins, lower-case for the cells.

On the IIKC scheme, light green area points out what to observe. The presence of a spot (pale or dark depending on the definition of the observed character) is represented by a red star (presence of a spot in the neighbourhood of the red star).

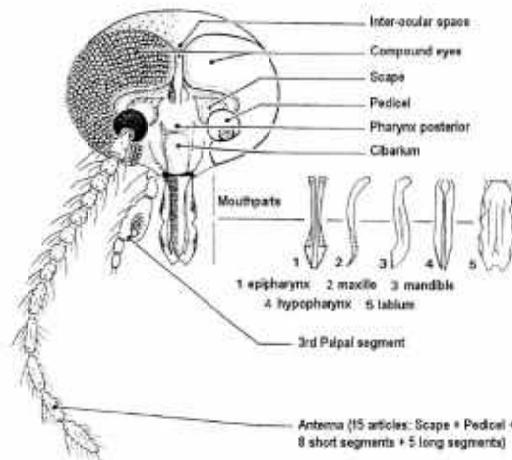


For example, the presence of a spot in the proximal part of the m1 cell is represented like the following:



- Head

Female Culicoides head



To evaluate the **shape of the sensory pits on the palp**, you have to check all around the pit using the fine focusing control of the microscope. If you don't, you can erroneously interpret an irregular shape as a regular one.

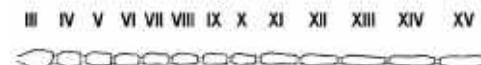
Culicoides antenna is compound of 15 segments. The first one, the scape, is ring-like and is hidden by the greatly enlarged second one, the pedicel. Antenna flagellomeres started from the antennal segment III to XV. On female, the 8 firsts (from III to X) are shorter than the 5 lasts (from XI to XV segments are elongated).

It's more and more frequent that taxonomists consider describing the flagellum, exiting the scape and the pedicel.

Thus, the correspondence between flagellomeres and antennal segments is given in the following table:

| Flagellomeres | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|-------------------|-----|----|---|----|-----|------|----|---|----|-----|------|-----|----|
| Antennal segments | III | IV | V | VI | VII | VIII | IX | X | XI | XII | XIII | XIV | XV |

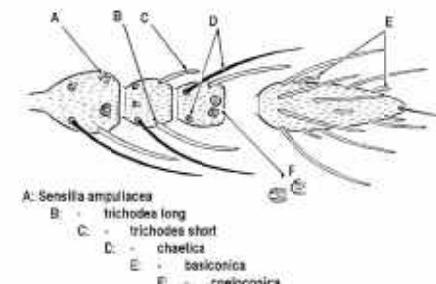
Antenna



Antennal ratio "XI/X ratio" is the length of the first elongated segment (XI) divided by the last short segment (X). Here, only an estimate of the ratio is asked (no measure required).

The sensilli present on flagellomeres are represented on the following drawing:

SENSILLI



In the key IIKC, the sensilla coeloconica (F), the long trichodea (B) and the short blunt tipped trichodea (C) are used as taxonomic descriptors.

It's important to observe the **sensilli coeloconica distribution (SD)** on both antennae. If sensilli are present or absent on 1 segment of both antennae, you can consider the SD with confidence. In case of a SD disagreement on a segment of both antennae, most of the time, it means presence with an abnormal sensilli absence on a segment. Thus, in that case, you can select "presence" to the appropriate state of the descriptor.

In the IIKC, this SD is divided in 3 descriptors (from III to VI, from VII to X and from XI to XV). These descriptors are powerful but if you feel the SD variable according the observation of both antennae, it would be careful to not use it. In that case, to reduce the error possibilities, you can use the others descriptors to reduce the taxa list and try the SD later.

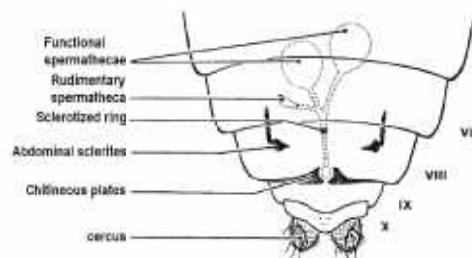
The **short trichodea sensilli** are not easy to observe. Sometimes the species with 1 short trichodea per short flagellomeres, can present an extra second one on few segments. In that case, the species are considered as 1 seta per segment.

The **long trichodea sensilli** are used only for either their enlarged or regular aspect. This observation is quite easy.

The **sensilli ampullacea, chaetica and basiconica** are not used in this key.

- Abdomen

General view of the last abdominal segments of female

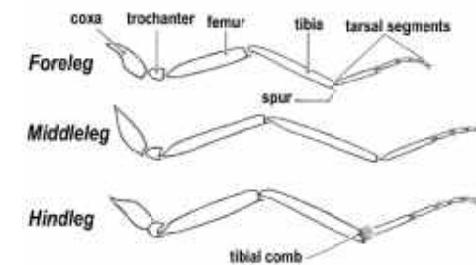


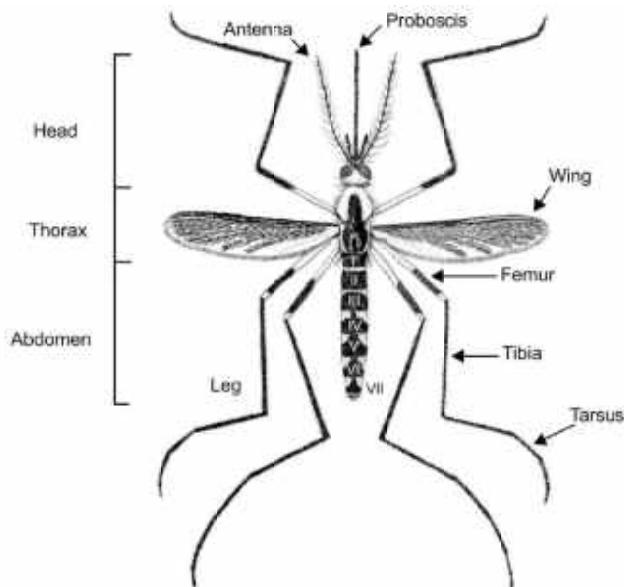
The presence of a **sclerotized ring** is an important morphological character, don't forget to check it! It's an easy step to split the *Culicoides* species in two groups.

Some parallel ones can be difficult to observe even with a clear abdomen. To help to sort this out, you can follow the spermathecae duct by moving the fine focusing control of the microscope and proceed through the genital opening.

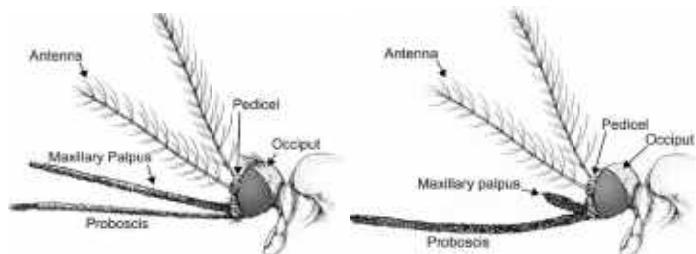
- Legs

The only difficulty is to avoid interpreting a seta wider than the others like a spine. How to recognize a fore, middle or hind leg ? Foretibia present a spur well developed. Hindtibia present a comb of 2 rows of spines while midtibia present no particular structure.



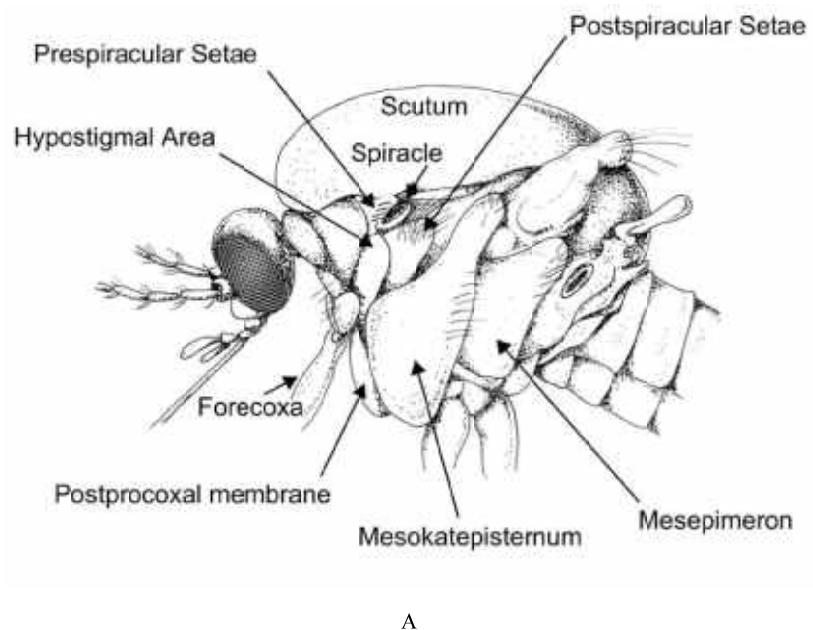
Adult Morphology

A

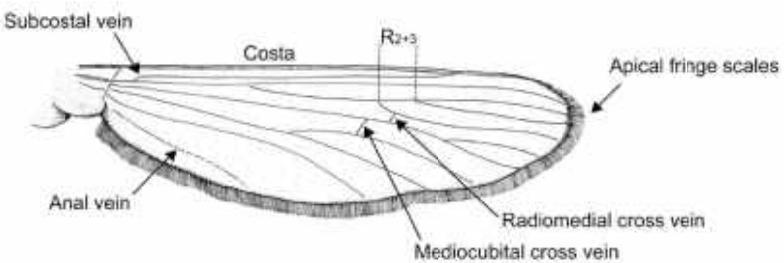


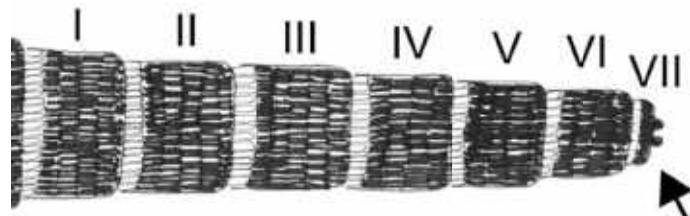
B

C

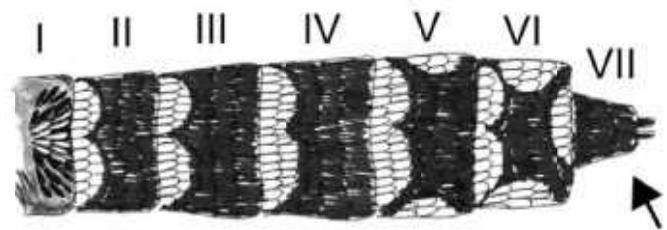
Plate 3. A. Adult; B. Anopheline head; C. Culicine head

A

Plate 4. A. Lateral view of thorax**Plate 5.** Wing;

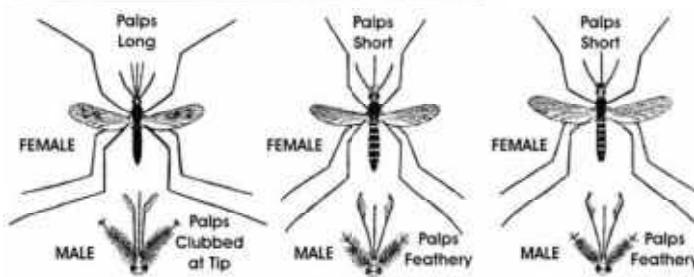
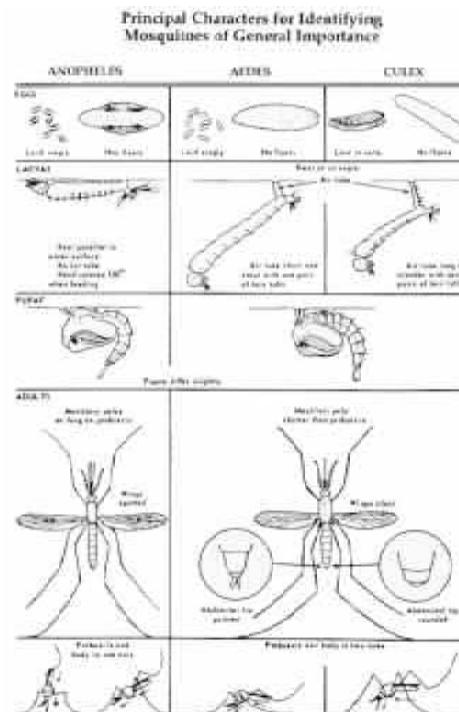


B



C

Plate 5. B. Blunt abdomen; C. Pointed abdomen



Anopheles

Aedes

Culex

IICK - Interactive Identification Key for *Culicoides* females from the Western Palaearctic region

Introduction

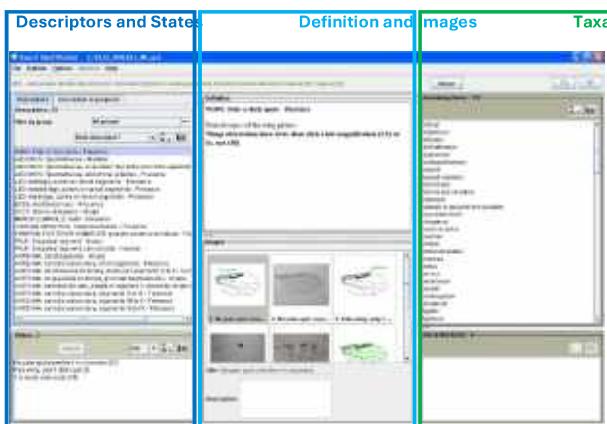
There are currently 110 taxa unit (100 species and 10 morphological variations) of biting midges *Culicoides* (Diptera: Ceratopogonidae) from the West Palaearctic region. 75 Taxa are illustrated with 599 photographs and 72 with drawings in PDF file. Distribution map in the West Palaearctic area is given for each taxa unit. Morphological characters are illustrated with scheme and photographs. This IICK is richly illustrated with a total of 953 photographs, scheme, drawings and maps.

How to use IICK?

The Xper2 platform is available in a few languages (English, French, Spanish, Chinese and Portuguese). Select in the "Options" menu, "General" and then "Languages". However, the database IICK is only available in English.

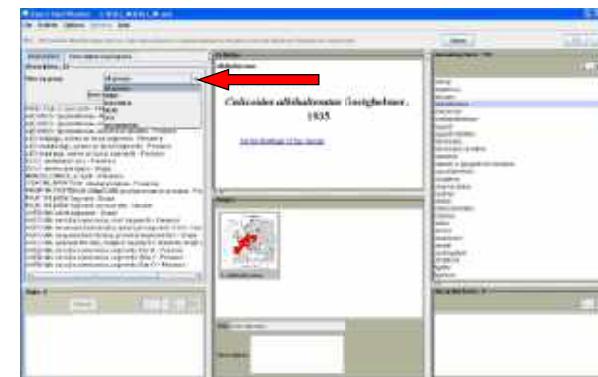
1. General view of the key

When running the identification, the remaining taxa are shown in the upper green window whereas the discarded ones in the lower green window. The list of descriptors available and their states are shown in the upper and lower blue windows respectively. All lists are updated after each descriptor state selection.

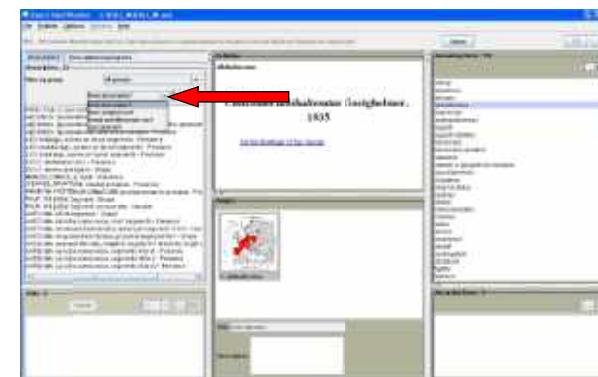


2. How to carry on the identification?

IICK is a multi-entry key therefore you can select a group of descriptors (wing, abdomen, leg, head, or geography). However, we recommend the use of "All groups" (default).



IICK allows short and quick identifications by classifying the descriptors according to their discriminating power. We recommend the use of the "Xper original sort". When activated, a number into brackets appears for each descriptor (from 0 to 1) representing the discriminating power. Highest numbers are the most powerful descriptors.

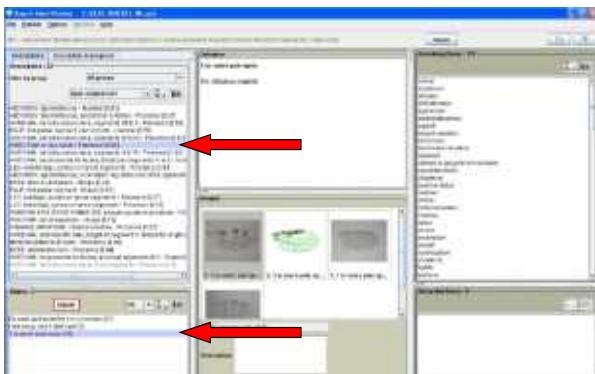


Start the identification by choosing a descriptor you want to work with and select the state you observe. Then click on the "Submit" button. For a multiple selection, press the Ctrl button to



select several states. At any moment you can **enlarge the pictures** by a double click on the left mouse button.

An operator list permit you to select XOR, AND, NOR, NXOR, etc. We do not recommend the use of operators other than "OR" (default).



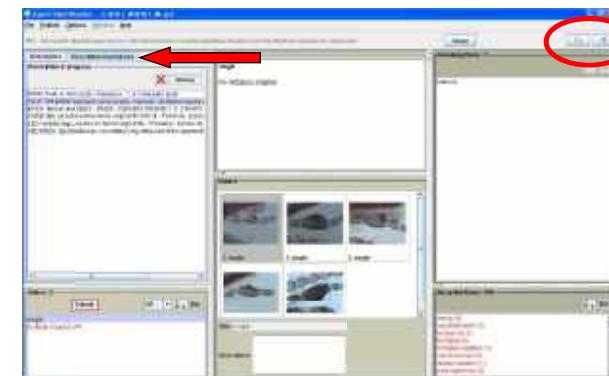
To start a new identification, click on the "Reset" button to initialize the IICKC. The classification according the "Xper original sort" has to be selected again.

3. Some useful tools:

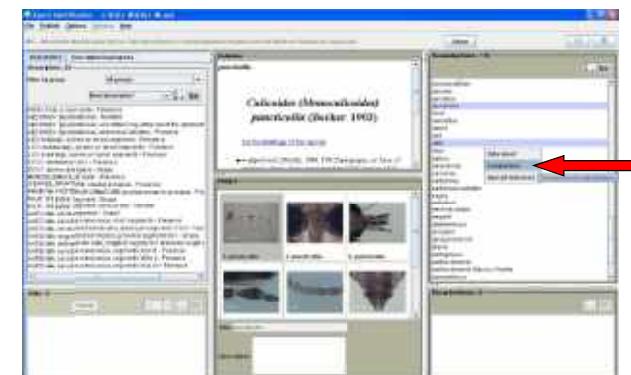
At any moment during the identification, you can check

- your progress (go to "Description in progress" tab),
- select or delete any steps (use the red cross button),
- modify your selection (change the selected state and click on "Submit"),
- generate an history of the identification (go to the "History" button).

At the upper right corner, the left button generates the database properties. The right button opens a help file called "**Help text with some taxonomic recommendations for a correct use of the IICKC**".



IICKC allows the comparison of different taxa. Press the Ctrl button and the left button on mouse to select the taxa you want to compare. Then click right button on mouse and select "Comparison".



preparation of sporozoites, the salivary glands should be ruptured by a gentle pressure of a needle, and they can be slightly mixed with a minute drop of the normal saline to produce thin small films. The preparation then is dried in the air, fixed with methanol, and stained the same way as blood smears.

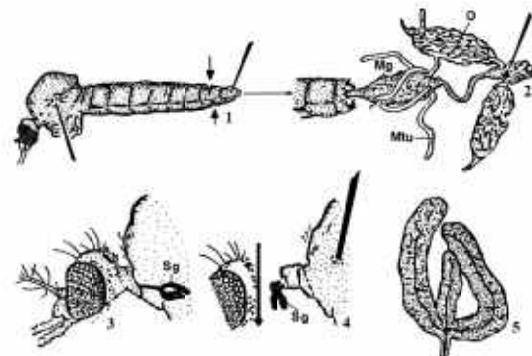


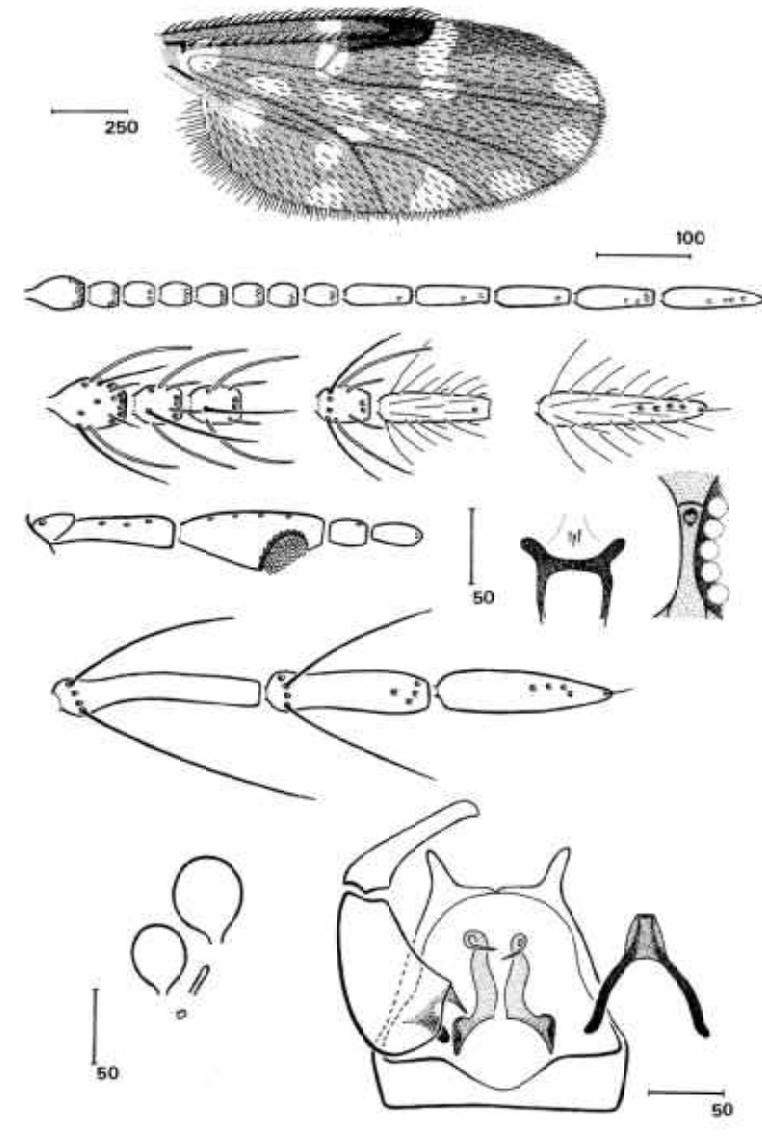
Figure 4. Successive stages of dissection of mosquito females for isolation of the midgut (1, 2) and salivary glands (3–5): 5 – isolated salivary glands; Mg – midgut; Mtu – Malpighian tubule; O – ovary; Sg – salivary glands (modified from Service, 1993). Source; Valkiūnas, 2005.

Preparations of gametes, zygotes, and ookinete (exflagellation)

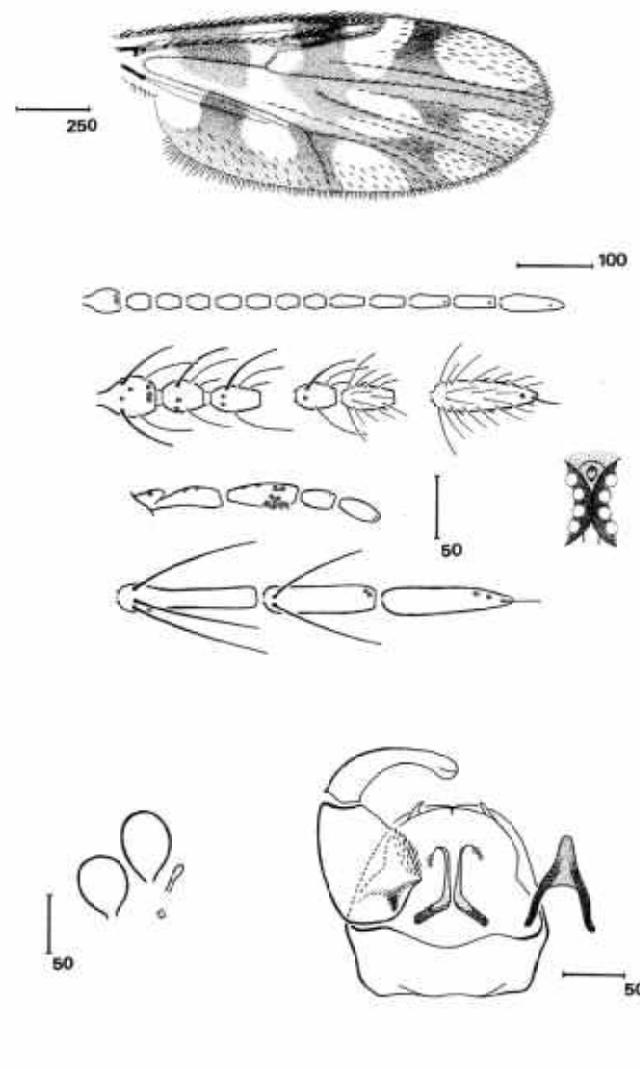
Formation of gametes, zygotes, and ookinete of many species of bird haemosporidian is easily induced *in vitro*, if the blood containing mature gametocytes is exposed in the air. The blood of infected birds is taken either from the heart or from the vein, then quickly put it on a watch glass and diluted with 3.7% solution of sodium citrate in a relation of 1 part of the solution by 4 parts of blood. The smears are prepared similarly as described above at each equal interval of time after the blood exposure in the air (1, 3, 5, 10, 15, 30, 45 min and 1, 1.5, 3, 6, 12, 24, 48 h). They are dried in the air, fixed with methanol, and stained with Giemsa's stain as described above. To prevent drying of the solution with blood the watch glass is placed in a Petri dish with a sheet of filter paper wetted in water on its bottom. The work is performed at 18–20°C. Exflagellation, fertilization and formation of ookinete are induced this way most easily in the species of *Haemoproteus* and *Leucocytozoon*, while in some species of *Plasmodium* it is induced not so easily and require using special media (for example, those containing blood serum). In the case of *Plasmodium*, this process is easily induced by infecting mosquitoes, which are later dissected after a certain period. This method also gives good results with parasites belonging to all genera of bird Haemosporida.

Source: Valkiūnas, G. (2005) Avian malaria parasites and other haemosporidia. CRC Press.

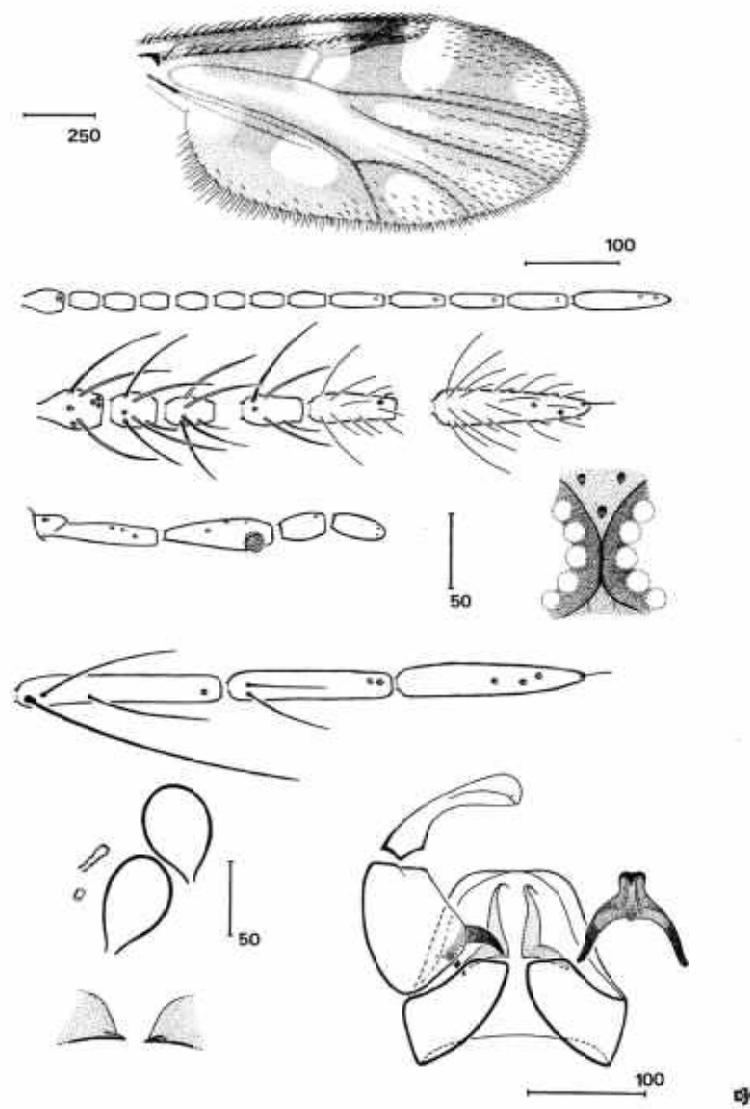
Culicoides festivipennis



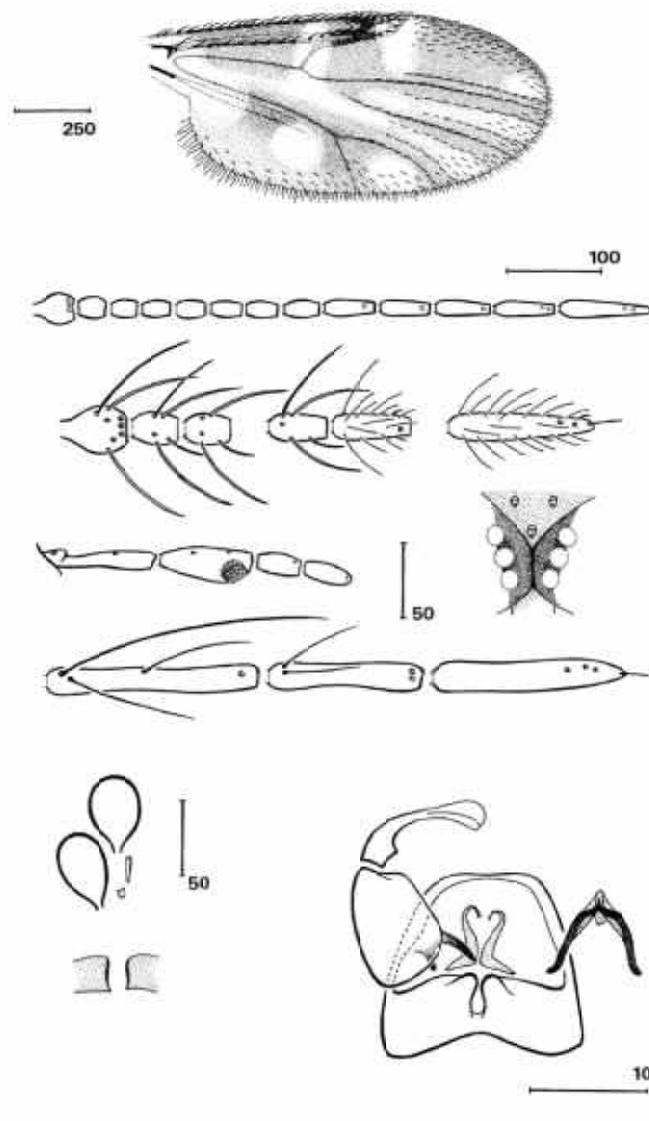
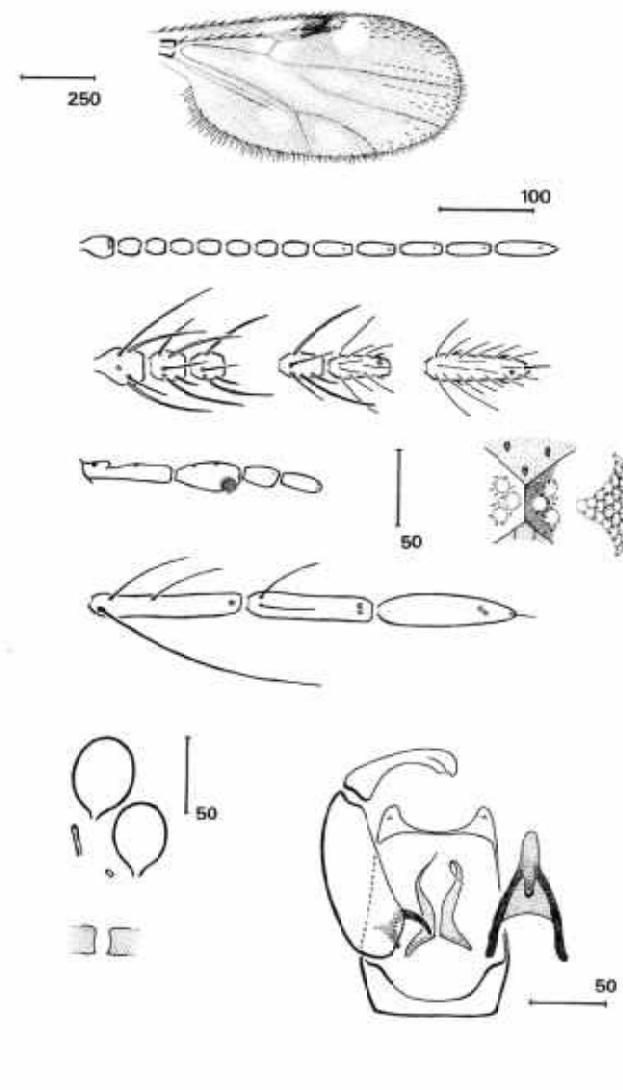
Source: IIKC Interactive Identification Key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palearctic region

Culicoides impunctatus

Source: IIKC Interactive Identification Key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palearctic region

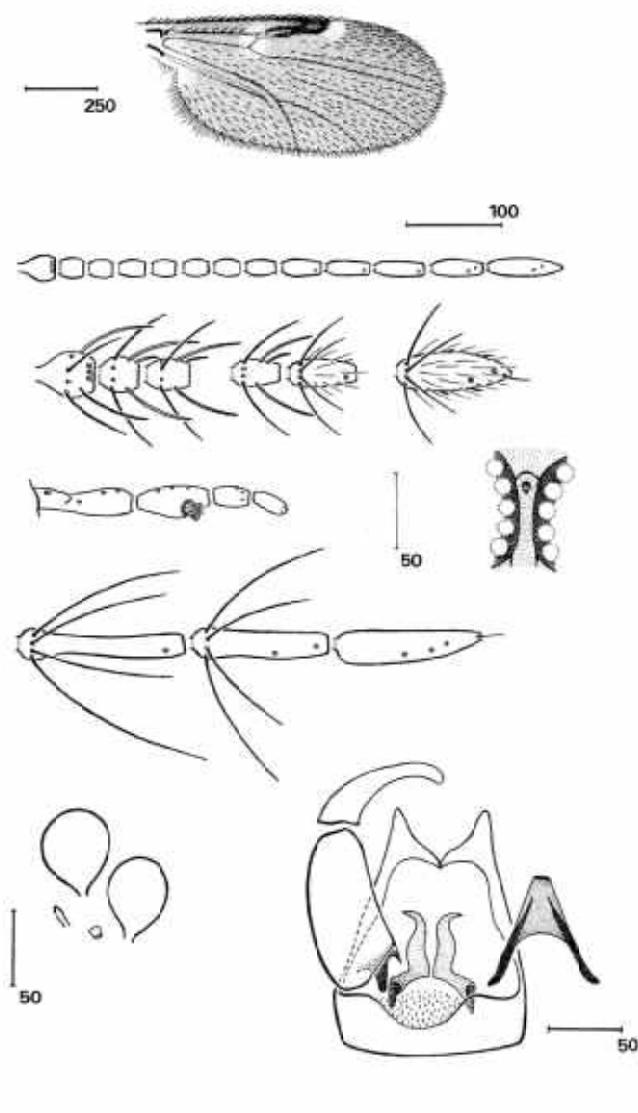
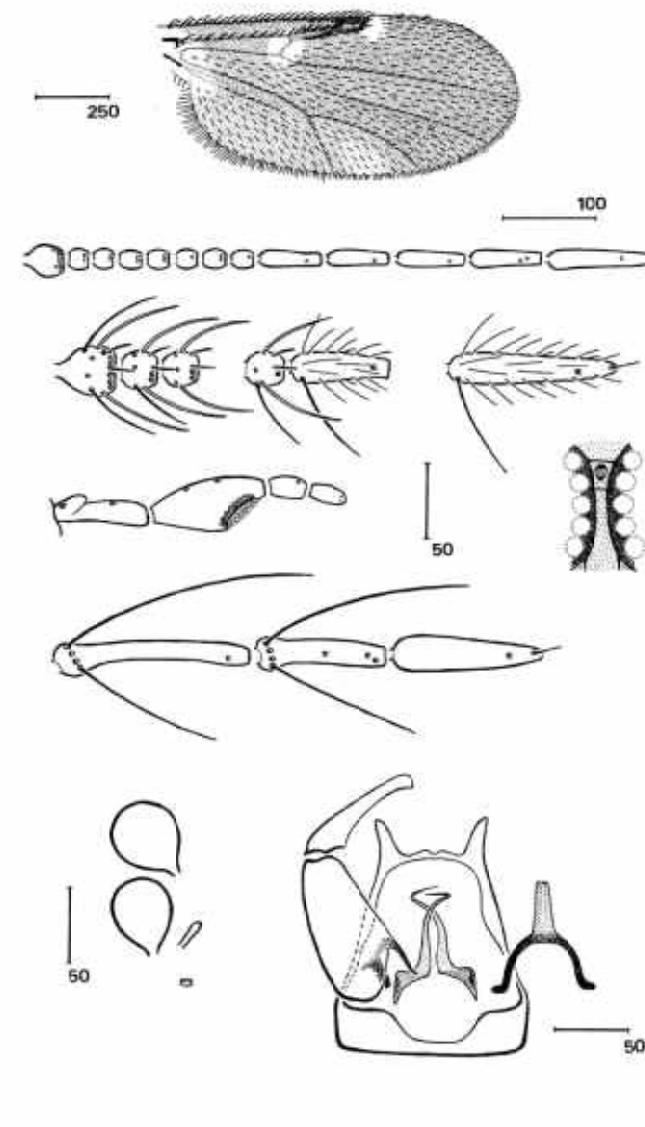
Culicoides scoticus

Source: IIKC Interactive Identification Key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palearctic region

Culicoides obsoletus*Culicoides chiopterus*

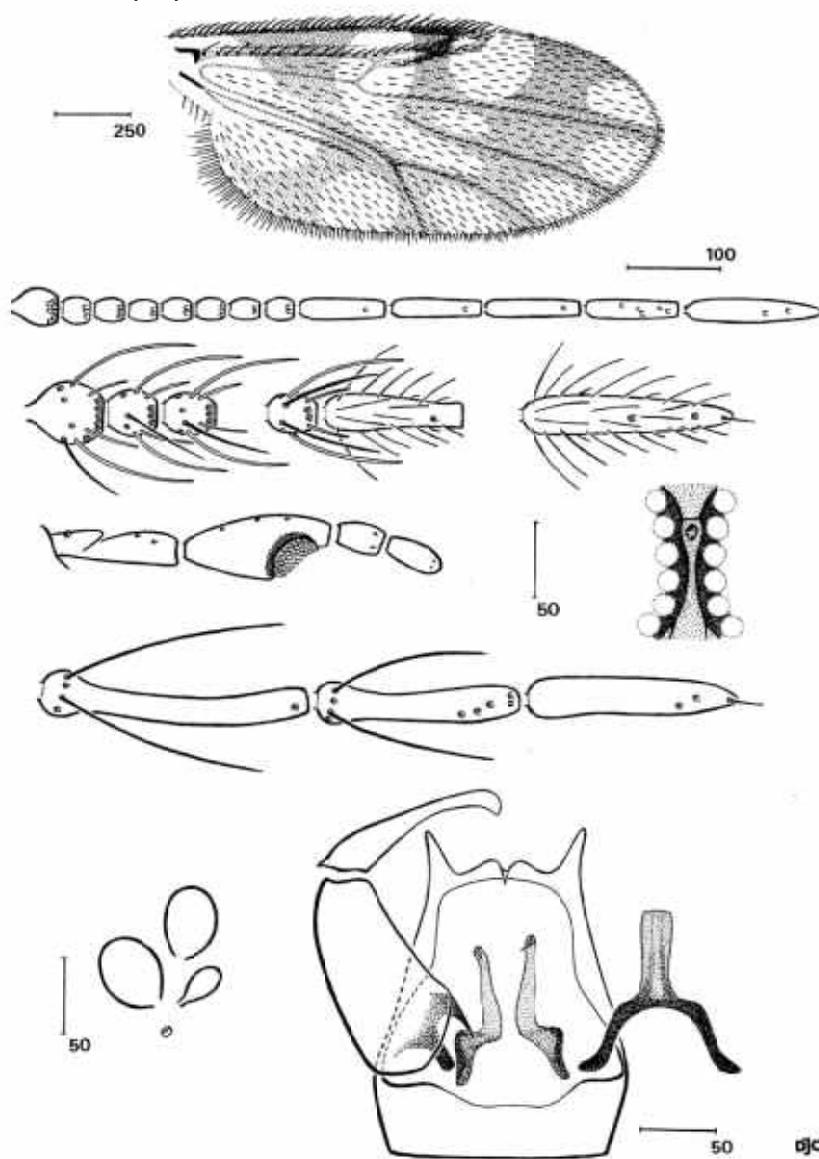
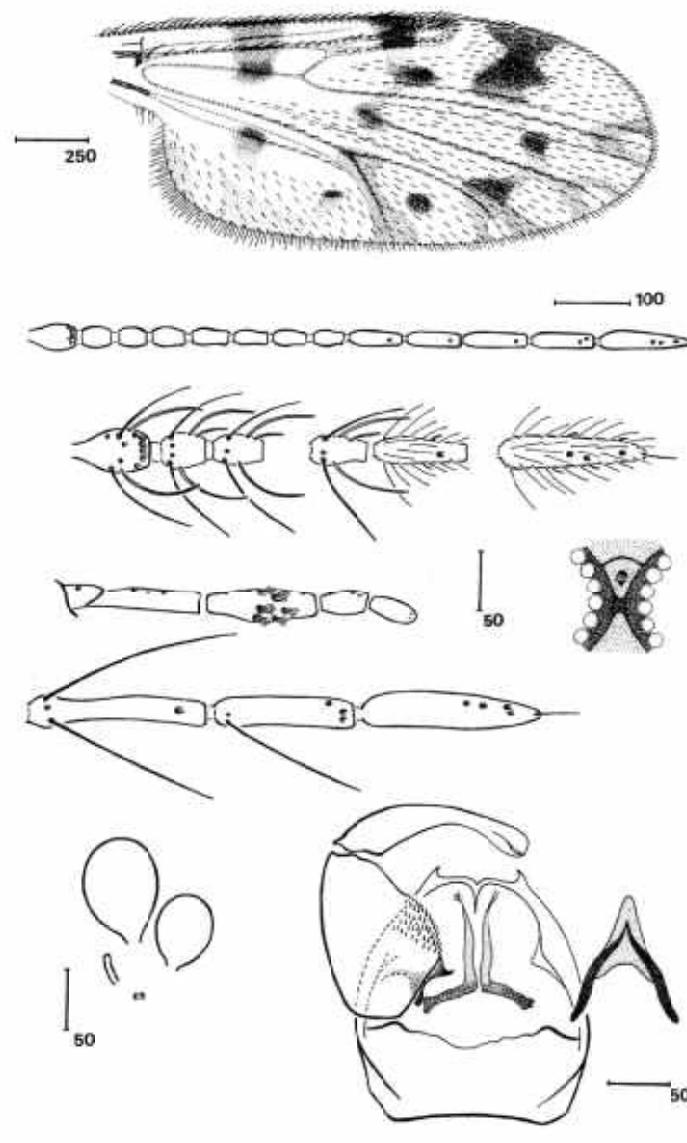
Source: IIKC Interactive Identification Key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palearctic region

Source: IIKC Interactive Identification Key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palearctic region

Culicoides pallidicornis*Culicoides kibunensis*

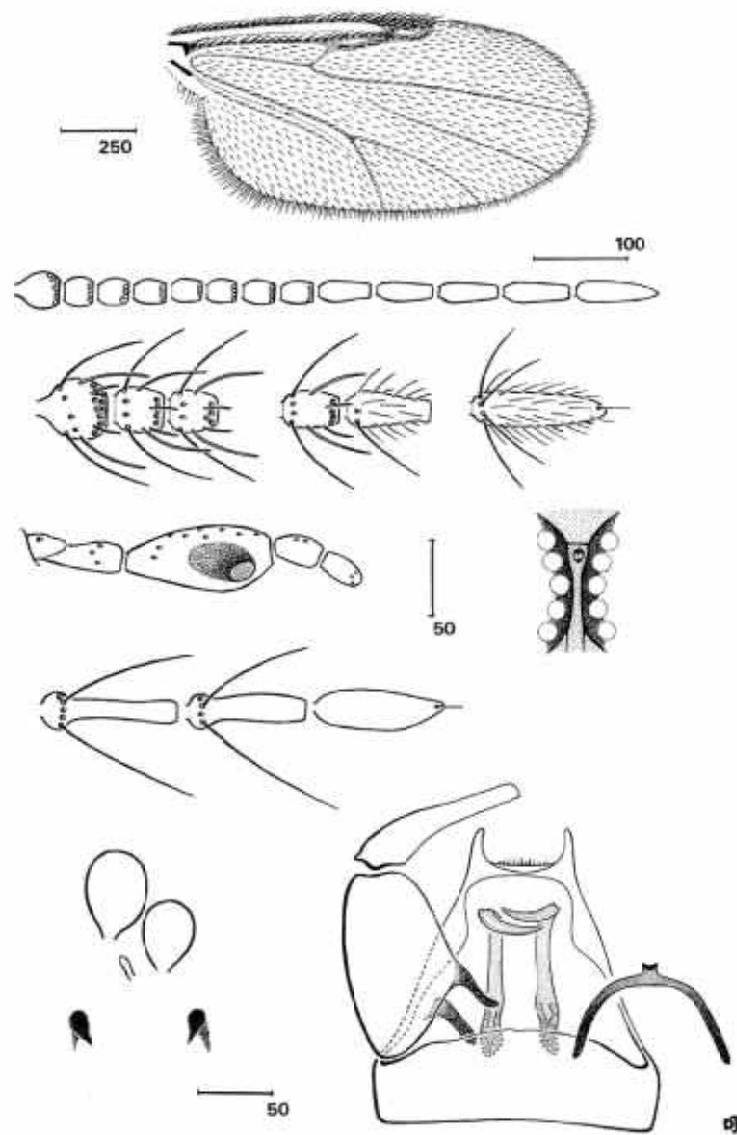
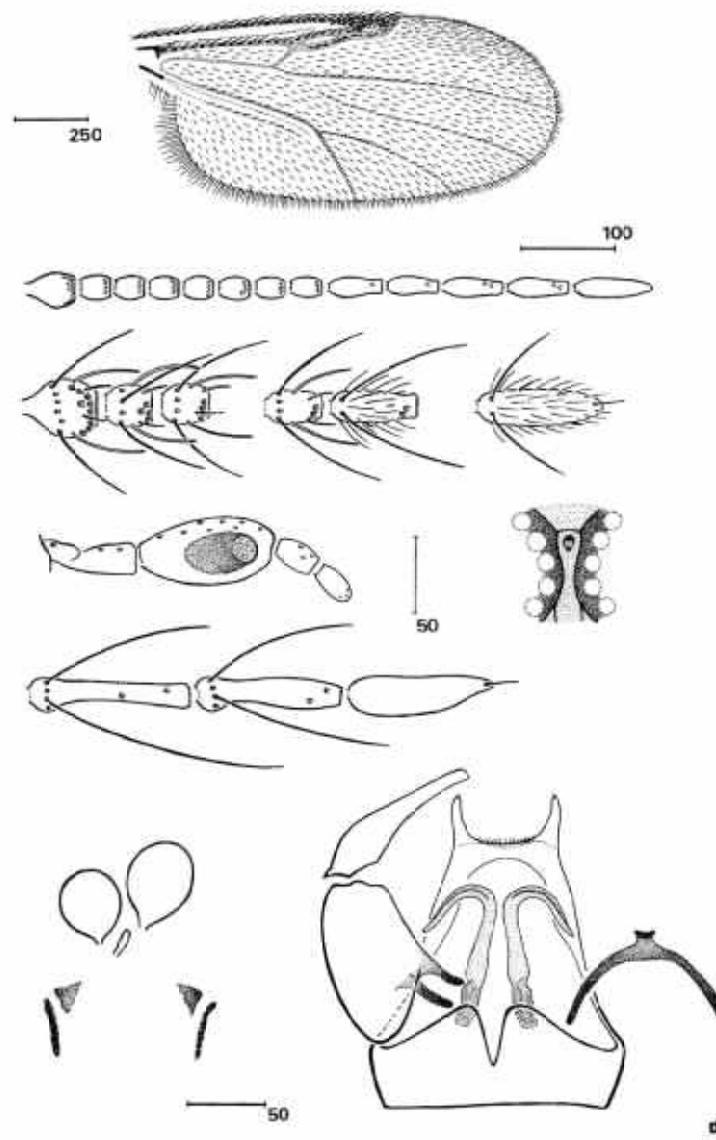
Source: IIKC Interactive Identification Key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palearctic region

Source: IIKC Interactive Identification Key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palearctic region

Culicoides pictipennis*Culicoides punctatus*

Source: IIKC Interactive Identification Key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palearctic region

Source: IIKC Interactive Identification Key for Culicoides (Diptera: Ceratopogonidae) females from the Western Palearctic region

Culicoides reconditus*Culicoides segnis*

SEQUENCE ALIGNMENT EXERCISE

For this exercise we have chosen the program Geneious (<https://www.geneious.com/>). It is a very dynamic program to a reasonable price (\$195 per year for students) and with many plugins. If you don't have a license, install the 14-day free trial version for Geneious Pro. Open Geneious as administrator (right-click program icon (in your start menu or on your desktop), Run as administrator). When the program has opened, select **Tools > Preferences > General**. You may now want to adjust how much RAM memory you allow Geneious. You can probably allow it 60–75 % of your total RAM. (If you don't know how much RAM you got, on a Windows computer you can find out by opening the start menu, then right-click on Computer, and select Properties.) Click **Ok**, and close Geneious, then open it again, for the memory settings to take effect.

Choose **Tools > Plugins**. Here you can select from a bunch of (free) plugins. We may use a few of them, so install **MAFFT multiple aligner**, **PhyML**, **Heterozygotes**, and **Mauve Plugin**. Click **Ok**. The installations can run in the background while you work on the exercises.

1. Start with the 3 sequence output files from the ABI instrument. (Detailed information will be shown during exercise).
2. The PCR was done with the HaemF (5'-ATGGTGCTTCGATATAATGCATG-3') and HaemR (5'-GCATTATCTGGATGTGATAATGGT-3'), the inner primer pair for nested PCR.
3. The sequencing reaction was done either from one end (the HaemF primer) or from both ends (you need to align and concatenate the two sequences).
4. Edit the sequences as explained in the instruction. Make sure to delete the 5' part of the sequence with unstable and ambiguous reading. Replace misread base-calls by the correct nucleotide, N for unknown, and other letters if the signal is clearly "double" (e.g. C & T = Y; see IUPAC ambiguity code table below).
5. Trim the primer sequence.
6. For those sequenced from both ends, align them, and choose the clean one for each site.
7. BLAST the obtained sequences to Genbank and MaAvi.

Question 1. What organism/gene have you sequenced?

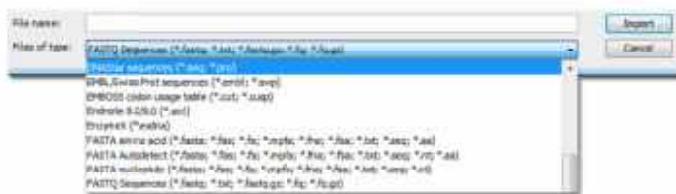
Question 2. What could be the reason for the repeated double base-calling in one of the sequences?

When you have answered the questions - proceed with editing a few more sequences, and when you feel comfortable with process, finish all sequences.

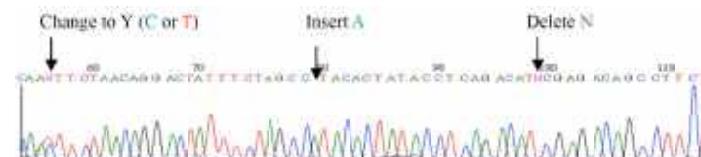
Aligning sequences in Geneious

Before you start, make a Geneious folder under Local (in the folder tree) by rightclicking on Local and selecting **New Folder**. Name it Workshop or something of your choice.

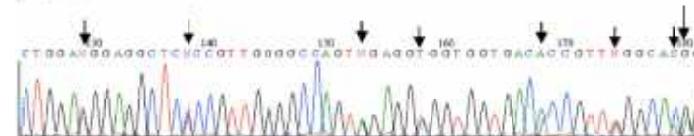
1. Create a new Geneious folder in Geneious (under Local) by right-clicking on **Local > New Folder**.
2. Import the sequence files into Geneious by drag-and-dropping. [It can also be done from Geneious by **Edit > Import > From File** or **Ctrl+I**.] When asked whether to **create a list** or **keep sequences separate**, choose separate.



3. Look at the abi sequence trace with chromatogram, by highlighting it or double clicking to open in a new window. The automatic interpretation of the chromatogram is not always correct. This occurs mainly when the sequence is "unclean" (high level of background), when there are two peaks at one position. By looking at your chromatogram you will be able to detect and correct the errors. Look for double peaks and weak signals. Undetermined nucleotides have been assigned by "N". Check all "N" and see if it is possible to assign the correct nucleotide by eye. Remember to click **Allow Editing**. You can always keep track on your editions.



Examples of errors in the chromatogram. In the sequence above, there are three errors to correct.



Double Sequence. In this example, two, or more alleles/haplotypes have been amplified.

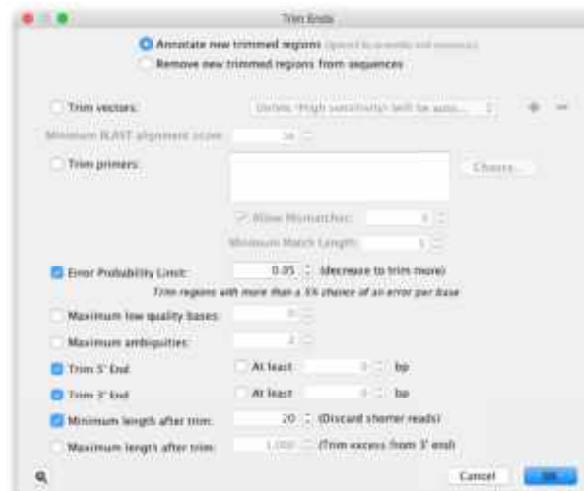
IUPAC ambiguity code table

| Code | Represents | Complement |
|------|-----------------|------------|
| A | Adenine | T |
| G | Guanine | C |
| C | Cytosine | G |
| T | Thymine | A |
| Y | Purine (C or T) | R |
| R | Purine (A or G) | Y |
| W | weak (A or T) | W |
| S | strong (G or C) | S |
| K | keto (T or G) | M |
| M | amine (C or A) | K |
| D | A, G, T (not C) | H |
| V | A, C, G (not T) | B |
| H | A, C, T (not G) | D |
| B | C, G, T (not A) | V |
| X/N | any base | X/N |
| - | Gap | - |

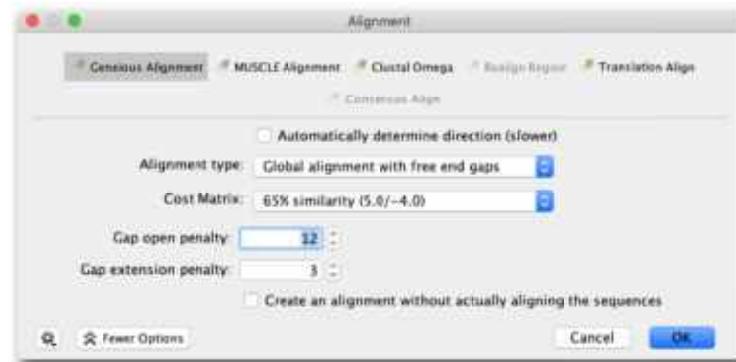
4. Trim the ends by **Annotate & Predict > Trim Ends** [Right-click > Trim Ends]. Select standard settings (Annotate new trimmed regions rather than Remove), click Ok. Trimmed regions will be annotated with the pink Trimmed annotation.



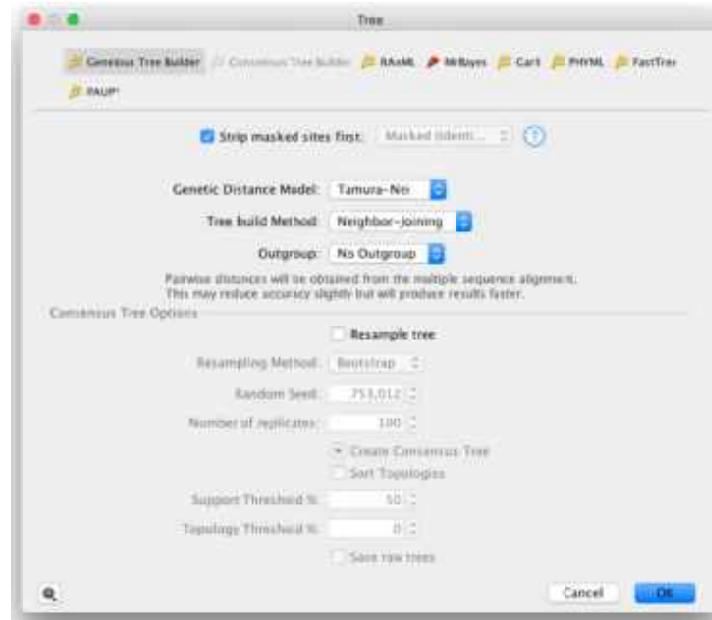
5. The end of the sequence represents a reverse primer, so let's extend the trimming of the 3'-end by
- Selecting the pink Trimmed annotation by clicking on it.
 - Hover with the mouse over the left end of the annotation until a doubleended horizontal arrow turns up.
 - Click and drag the end, to extend the Trimmed annotation so that it covers the HaemR2 primer.
6. Possibly repeat for the 5'-end if you think more ambiguous peaks need to be trimmed out. You can also import the primer sequences to the local database and click **Trim primers**.



7. If one sample was sequenced from both ends, you need to reverse complement the reverse one, i.e. reverse the sequence direction and replace each base by its complement. Click the R.C button above the sequence viewer, or go to **Reverse Complement** under the **Sequence** menu. Align the two sequences by clicking **Align/Assemble > Pairwise align**. Use the **Geneious Alignment** algorithm with standard settings. (In case you forgot to reverse complement the reverse sequence, click **Automatically determine directions**) If there are disagreements between the two, keep the clean one (clear single peak in one position), although this may lead to an underestimate of mixed infection. Save the changes in the sequence by hitting **Save** or **Ctrl+S**.



8. When all sequences are done, make an alignment by clicking Align/Assemble > Multiple Align [Ctrl + Shift + A].
9. Rename the alignment by selecting it and clicking again on the name (slow double-click) [or hit F2]. Name it Malaria1 for example.
10. Open the alignment (double-click) in a new window.
11. Using your mouse and/or ShiftSelect the part of the lab sequence (with chromatogram) which is not trimmed. (Zoom in if you need.)
12. Select this region from all sequences by extending the current selection: Hold the Shift button and use the down arrow or PgDn to extend the selection downwards over the remaining sequences.
13. Click Extract > Extract region as alignment. Untick "Modify sequence names..." .
14. Close the Malaria1 alignment and return to the main window (you may be referred to the main window automatically, but then the old alignment window is still active in the background, and you could preferably close it). In the main window, you should now also find the Malaria1 extraction.
15. Select it and hit Tree. The simplest tree will be made through Geneious Tree Builder, making a NJ tree. Choose one of the sequences as outgroup and tick Resample tree with bootstrapping. You can also reconstruct a Bayesian tree using MrBayes plugin (it may take a while).



16. Open the resulting tree, and look at it in Tree View.
[If you had not chosen an outgroup, the tree can be rooted afterwards by clicking on any leaf node (so that it is marked with a blue circle) and choosing Root].
If you use Mybayes plugin, check whether the ESS exceeds 200.
17. You can BLAST the sequences directly from Geneious by selecting it and the hitting Sequence Search.
18. Choose the nr database from GenBank, and restrict the number of hits (Maximum Hits) to 5. Hit Search.



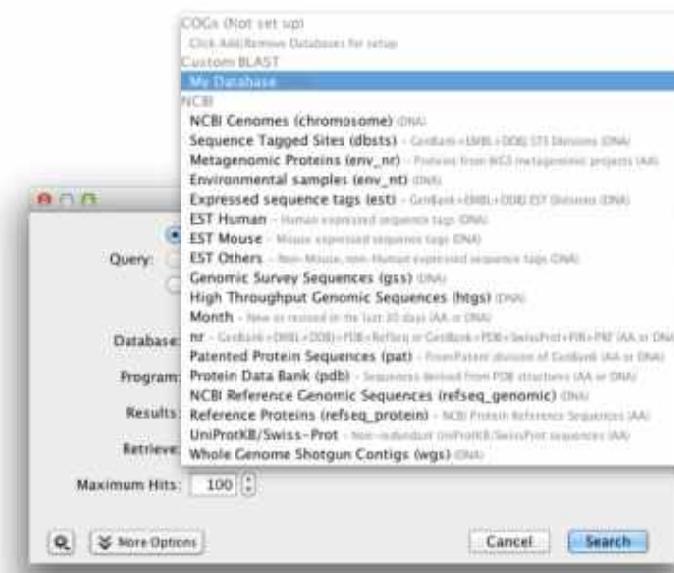
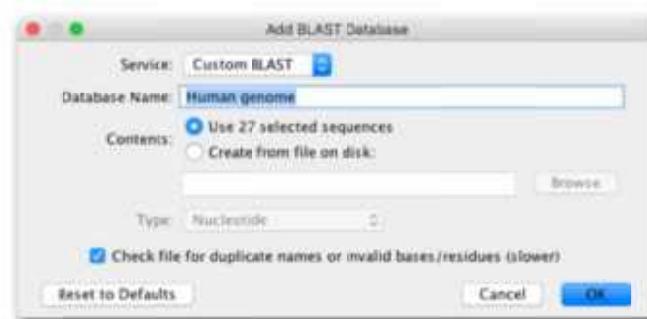
19. Your searches will turn up in a separate folder under Searches, in the Geneious folder structure. (The BLAST's against NCBI can be a bit slow!)

Search hits can also be sorted by other columns by clicking on the column header. Columns that may be useful to sort by include **E-value**, **Percent Identity**, **Query Coverage** or **Grade**. **E value** or "Expect value" represents the number of hits with at least this score that you would expect purely by chance, given the size of the database and query sequence. The lower the E-value, the more likely that the hit is real. You can export the result to a .csv file.

20. You can also use custom blast to blast your sequences against MalAvi. Firstly, download them from MalAvi website by clicking 'Fasta'. Then turn to Geneious, go to Tools → Add/Remove Data-bases → Add BLAST Database and select Custom BLAST from the Service drop-down box. Choose to Create from file on disk and then click Browse to navigate to the fasta file. Name the database 'malavi' and click 'OK'. Now you can blast your sequence. Compare the results against Genbank and MalAvi.

For clear double infections, try to separate the two lineages based on blast result (one lineage can be 100% identical to recorded lineages, the rest base in the mixed peak belongs to the other lineage).

Download the 'Host and Site' Table directly from MalAvi or use malaviR package, check host species and recorded region of the lineage.



One can also use Geneious to identify cases of mixed infections. Select all sequences, **Annotate & Predict > Find Heterozygotes**. There's no real point in looking in the regions that you already trimmed because they are of low quality (so do NOT tick Search in trimmed regions). Set the **Peak Similarity** to 50 %.

We also attached the protocol to use MEGA (download <http://www.megasoftware.net/>) because they are easy to use and freeware. You can follow the instructions to edit and align sequences, as well as blast and construct phylogenetic trees, feel free to explore the software on your own. For phylogenetic reconstruction there is an almost endless list of programs. PAUP, MrBayes, RaxML, Garli are among the most frequently used. We can also use various R packages for BLAST and phylogenetic analysis, such as malaviR, phytools, etc.

1. Instructions to edit, BLAST and align sequences using MEGA

1.1. Open MEGA. Open your first sequence file xxx.ab1 [Align / Edit/view Seq...].



1.2. The chromatogram is now shown. Now, you can edit your sequence by changing the letters when necessary.

1.3. The automatic interpretation of the chromatogram is not always correct. This occurs mainly when the sequence is “unclean” (high level of background), when there are two peaks at one position. The beginning and the end of the sequences often needs quite some trimming. By looking at your chromatogram you will be able to detect and correct the errors. Look for double peaks and weak signals. Undetermined nucleotides have been assigned by “N”. Check all “N” and see if it is possible to assign the correct nucleotide by eye. You can jump to the next “N” by using CTRL+N.

1.4. Find the primer sequence and delete it. Use CTRL+F

1.5. Save the edited sequence as xxx.scf. Use CTRL+S

1.6. Do a BLAST search to find matching sequences in GenBank. This can be done directly in MEGA (blast against Genbank)

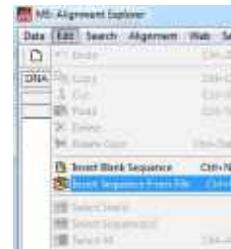
1.7. Or else you can use the BLAST module in MalAvi website or with malaviR package. Compare the BLAST results.

1.8. If the sequence appears to be simple double-infection (with very few mixed peaks), try to separate them based on BLAST result.

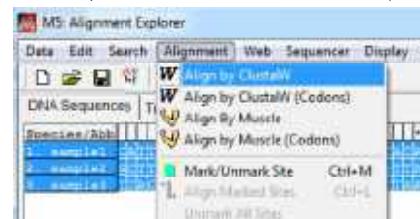
1.9. When you have edited and saved all the sequences as xxx.scf files, the next step is to add them to a common alignment, choose "Create a new alignment" and then "DNA"



1.10. Add your edited sequences to the alignment. Change to .scf format to find your files. You can mark them all and add them in one go.



1.11. These sequences might start at different positions (depending on how close to the forward primer you were able to read unambiguously). Therefore, these need to be aligned. Highlight the sequences and use ClustalW (and the suggested default settings)



1.12. For those sequenced from both ends, check the quality of both sequence before alignment, delete unclean parts. Define whether there is mixed infection based on the quality of both sequences.

1.13. Remember that this is a protein coding DNA sequence. Check the alignment for gaps. If you find gaps, this is most likely because of errors when reading the chromatograms (a missing or extra nucleotide will change the reading frame and make the protein useless). Go back to the chromatogram and find out how to remove the gaps.

1.14. Save your alignment xxx.mas

1.15. For phylogenetic analyses you may want add more distantly related taxa to be used as outgroups, or some other taxa inside the group of interest (for example mammalian *Plasmodium*). You can search Genbank for such sequences within MEGA

1.16. For example, write "Leucocytozoon cytochrome b" in the search box and click Go. To find what you want, you sometimes have to play around with the keywords. You can also use this box to search for a specific GenBank entry (e.g. AB542068) or several (AB542068 AB542069 AB542070 AB542071 AB542072 AB542073 etc).

This is useful if you want to download a number of sequences reported in a publication.

1.17. You can now click on any of the entries and find out more of the details. If you want to add the sequence to your alignment, click 

1.18 After adding additional sequences you have to re-align them again.

1.19 Save the alignment

1.20 Export the alignment to a fasta file (xxx.fas) for later use in the MalAvi database

2 Phylogenetic reconstructions using MEGA

2.1. Open your file [Open a File/Session] and answer the following questions as they appear:

- "How do you want to open the file?" Analyze
- "Nucleotide sequences" Yes
- "Protein-coding DNA" Yes
- Select genetic code "Invertebrate mitochondrial".

In order to look at the data:

- Click Data / Explore Active Data



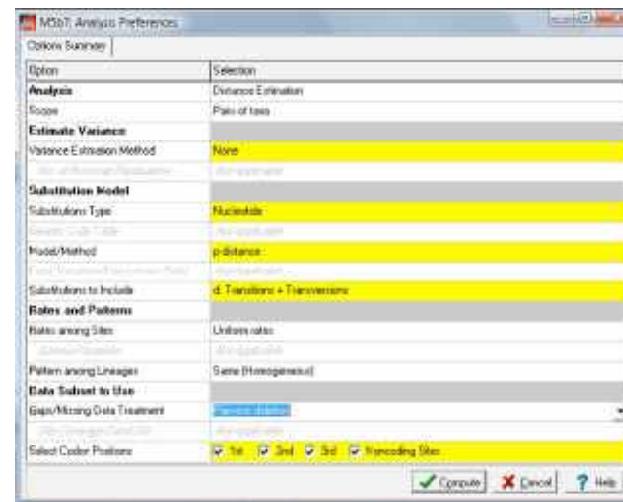
2.2. Explore the options: (and try to understand what these means)

- C – Conserved sites
- V – Variable sites
- Pi – Parsimony-informative sites
- S – Singleton sites
- 0 – 0-fold degenerate
- 2 – 2-fold degenerate
- 4 – 4-fold degenerate

2.3. Compute DNA distances

- In the menu, go to distance / compute pairwise distances

- In the Options summary, select "Model/Method = p-distance" and "Gaps/Missing Data Treatment = Pairwise deletions"
- Interpret the distances between the species
- Do this separately for Codon Positions 1st, 2nd and 3rd. Why are the distances not the same?
- Investigate the buttons C, V, Pi, etc



2.4. Compute amino acid distances

- In the menu, go to distance / compute pairwise distances and choose "Substitution type: Amino acid" and "Model/method = p-distance"
- Compare the distances with what you got from the DNA-distances

2.5. Neighbor-joining trees

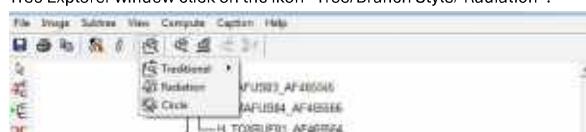
- Make a neighbor-joining tree:



- Choose the following settings (the option pair-wise deletion will use all the available data whereas the option complete deletion will only use the sites from where there is data from all sequences in the alignment):

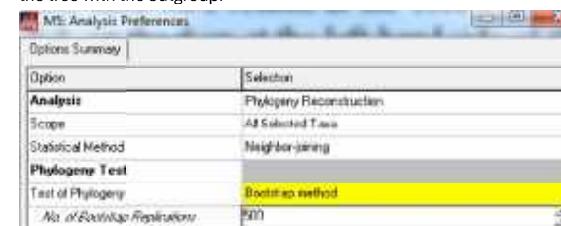


- A tree will appear in a separate window.
- Documentation and saving trees.** The most convenient way is to copy and paste trees into PowerPoint. Open a blank document in PowerPoint. In the "Tree Explorer" window of MEGA, select "Image/Copy to Clipboard". Jump to PowerPoint and "Edit/Paste".
- Rooting.** A tree will not show the phylogenetic relationships unless it is rooted. A more adequate representation of an unrooted tree is by a radiation diagram. In the Tree Explorer window click on the ikon "Tree/Branch Style/ Radiation".

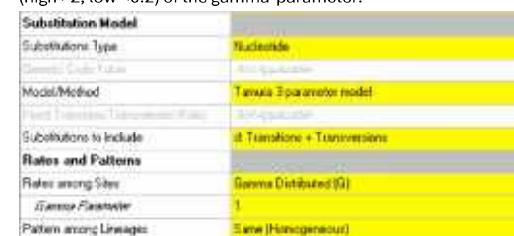


- Go back to the default representation "Tree/Branch Style / Traditional / Rectangular". To root a tree, click on a branch in the Tree Explorer window (now highlighted green) and then click on the ikon at the left-hand side of the window with the green triangle. (Try out different branches as roots).

- Bootstrap analysis** is a way to evaluate the accuracy of a tree, or rather, how well the obtained tree is supported by the data. Make a new neighbour-joining tree (as above) but now change "Test of Phylogeny" to "Bootstrap method". Press "Compute". Root the tree with the outgroup.



- Models of Molecular Evolution.** Try for example the Tamura 3-parameter model with rate variation between sites (see below for the settings). Try different values (high >2, low <0.2) of the gamma-parameter.



Question How do the trees differ (topology, support values, branch length)?

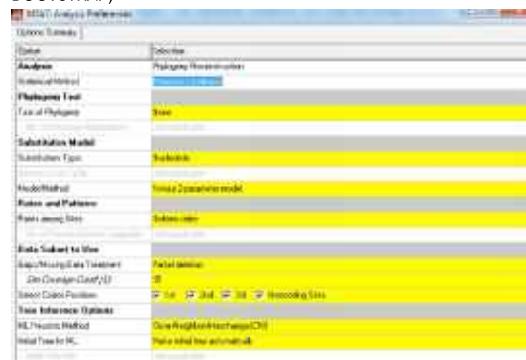
2.6. Maximum parsimony

One of the traditional and still very popular discrete method is Maximum Parsimony. Go to "Phylogeny/ Test Maximum Parsimony Trees". Use the default settings [[CNI (level=1) with initial tree by Random addition (10 reps)]] and select bootstrapping. Compare how much longer it takes to do bootstraps with this method compared to neighbor joining. Root the tree with the outgroup.

Character mapping. A nice feature with maximum parsimony is that the nucleotides can be mapped directly on the tree. In the Tree Explorer window, click on the upper-right symbol and choose "Show all". Walk through the sequence by changing the "Site Index"

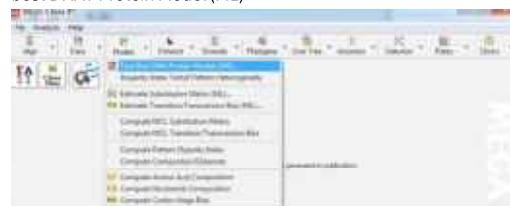
2.7. Maximum Likelihood

Go to "Phylogeny/ Test Maximum Likelihood Tree using the settings as below (DO NOT BOOTSTRAP)



Question. Compare this tree with the result from NJ analysis.

Find the most appropriate model of molecular evolution for your data. Go to Model/ Find the best DNA / Protein Model (ML)



Question. Study the output and try to interpret the parameters

Go to "Phylogeny/ Test Maximum Likelihood Tree. Using the settings as were found to be the most optimal model (DO NOT BOOTSTRAP)

Question. Are there any differences between this tree and the previous ML tree?

Now, make a final ML tree with bootstrapping (select 100 replications).

Question. Compare the bootstrap values with the NJ tree.