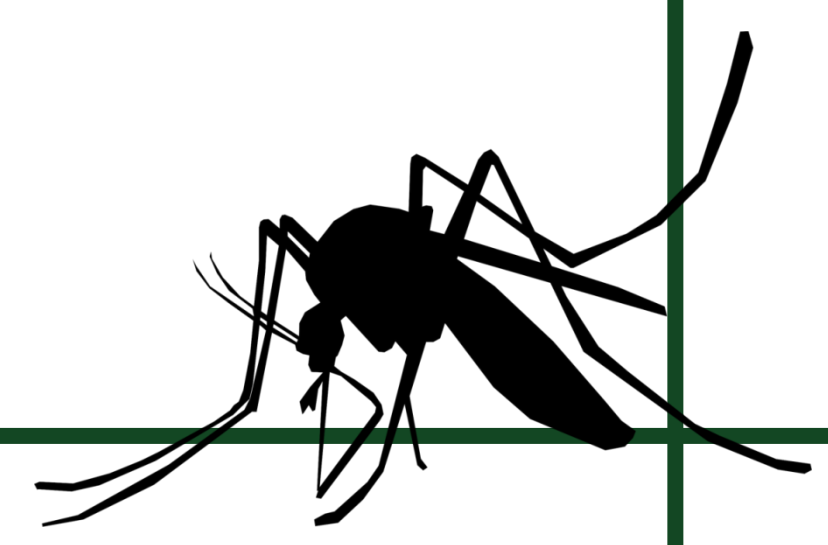# Monitoring of Exotic MOsquitoes in Belgium (MEMO)
Molecular validation of morphological species identifications

Nathalie Smitz[1,*], Isra Deblauwe[2], Wouter Dekoninck[3], Katrien De Wolf[2], Ingrid Verlé[2], Jacobus De Witte[2], Adwine Vanslembrouck[3],
Kenny Meganck[1], Sophie Gombeer[4], Yoo Ree Van Bourgonie[4], Marc De Meyer[1], Thierry Backeljau[4,5], Marc Coosemans[2] and Wim Van Bortel[2]

[1] BopCo, Royal Museum for Central Africa, Leuvensesteenweg 13, B-3080 Tervuren, Belgium.
[2] Unit of Medical Entomology, Institute of Tropical Medicine Antwerp, Nationalestraat 155, B-2000 Antwerp, Belgium.
[3] Scientific Service Heritage, Royal Belgian Institute of Natural Sciences, Vautierstraat 29, B-1000 Brussels, Belgium.
[4] BopCo, Royal Belgian Institute of Natural Sciences, Vautierstraat 29, B-1000 Brussels, Belgium.
[5] Evolutionary Ecology Group, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium.

* Corresponding author e-mail: nathalie.smitz@africamuseum.be

Due to globalization (trade and tourism) and global warming, novel introductions and establishments of exotic mosquito species (EMS) in Belgium are expected to occur, based on interception data and suitability models developed for some invasive species in Europe. The introduction of potential disease vector species constitutes a threat to human and animal health. In this context, the Belgian federal government and regional authorities are funding a monitoring project (MEMO) which aims at detecting and evaluating the occurrence of EMS in Belgium (focusing on six EMS with high invasive potential). The project is coordinated by the Institute of Tropical Medicine of Antwerp (ITM). Such species monitoring, however, requires reliable identifications. Within this framework, the Barcoding facility for Organisms and tissues of Policy COncern (BopCo project - the Belgian federal in-kind contribution to LifeWatch), which aims at facilitating identifications of biological samples of policy concern, will collaborate with the ITM and validate the morphological identification of a subset of samples using DNA-based methods. Therefore, the present contribution evaluates diagnostic DNA RFLP and sequence data for each mosquito species (N=37; native and potentially invasive) recorded in Belgium, and elaborates a workflow for the identification of the collected mosquito samples.

23 places where EMS are expected to enter Belgium (e.g. used tyre import companies, ports and airports), are monitored during three years (2017-2020). Eggs, larvae and adults are collected using various methods (see pictures). For each DNA marker, we evaluated its representativeness 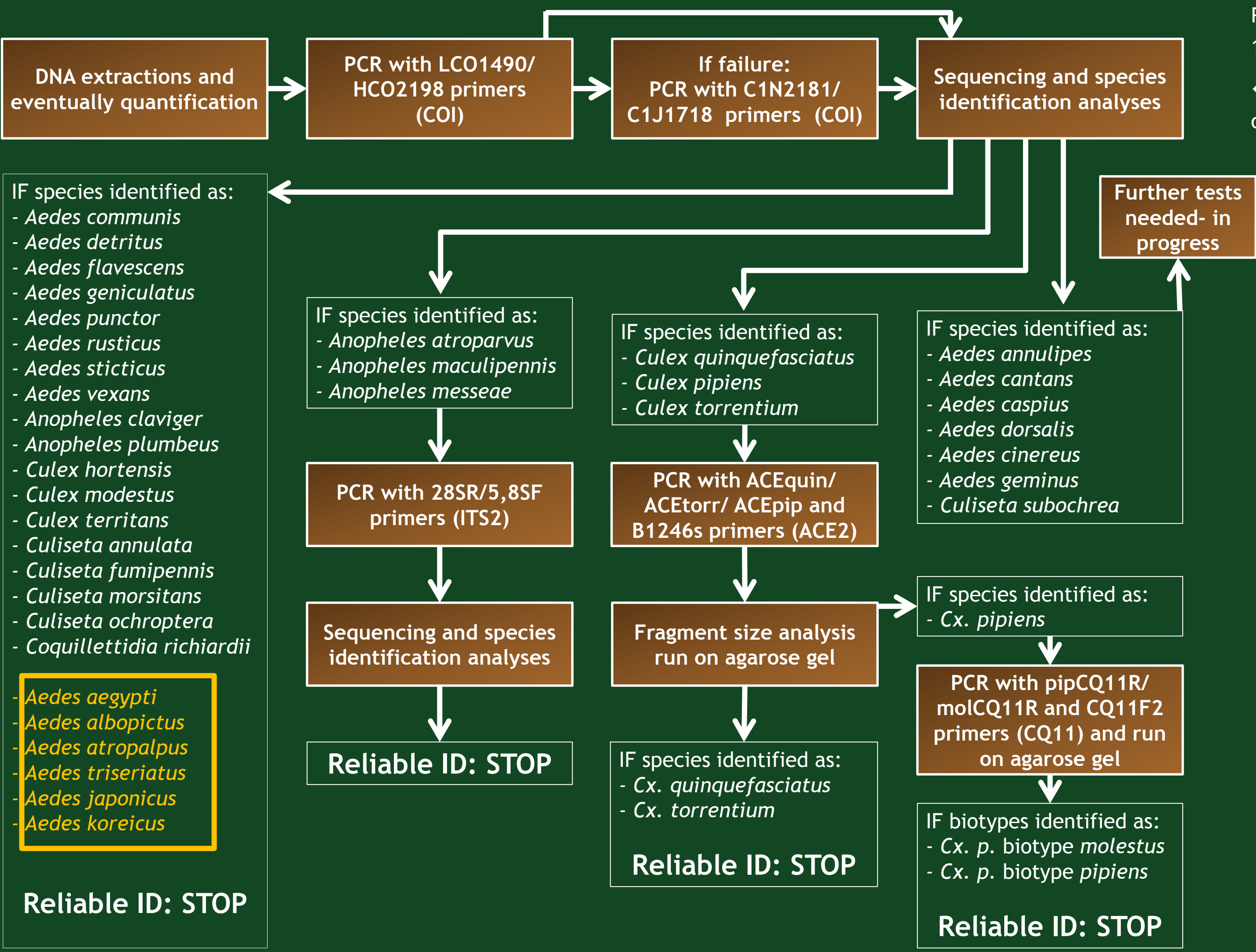on the online repositories BOLD and GenBank (i.e. number of reference sequences and geographical coverage) and its ability to provide a reliable identification for each of the 37 species. We then tested the laboratory protocols for the three best performing DNA markers (COI, ND4, ITS2) on 102 dried or ethanol preserved specimens (eggs, larvae or adults), representing 15 species.

Oviposition traps in a container and in a tyre © ITG

Mosquito Magnet Independence trap (propane bottle) © ITG

BG-Sentinel trap (Biogents) with batteries © ITG
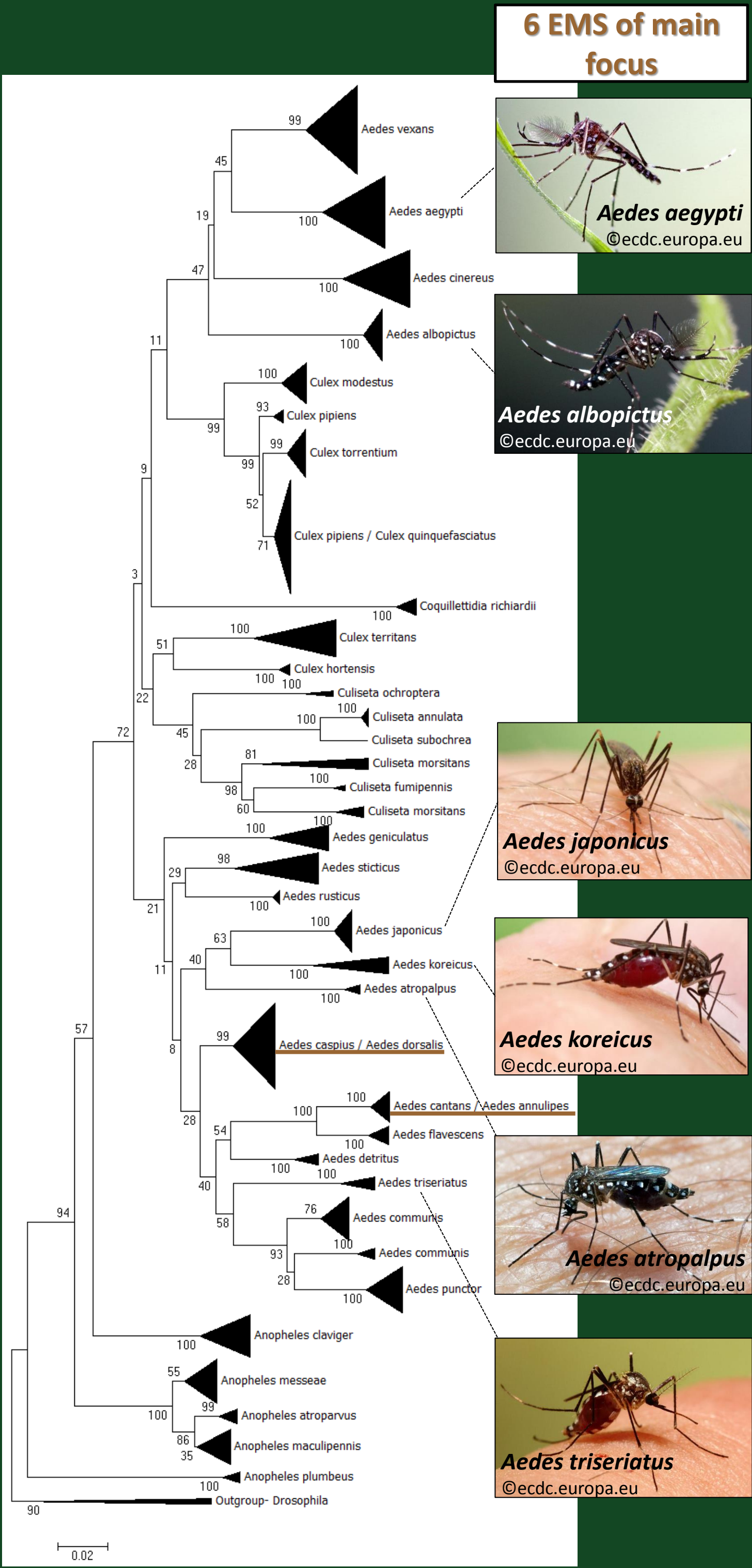
Each dataset (DNA sequences from the online repositories and new barcodes) was subject to NJ tree reconstruction (K2P, 500 BS) and DNA barcoding gap analysis. From the 37 mosquito species, 24 can be identified with high reliability using the COI Folmer region exclusively (65%), including all six EMS of main concern. For six other species (16%), additional DNA data are proposed in the identification workflow presented hereunder. In the case of the *Aedes caspius* / *Ae. dorsalis* and *Ae. cantans* / *Ae. annulipes* species complexes further study to develop specific identification techniques is required. A third species complex, *Ae. cinereus* / *Ae. geminus* cannot yet be resolved due to the lack of available material for *Ae. geminus*. Finally, for *Culiseta subochrea*, not enough DNA data could be retrieved ($N_{seq}= 1$).
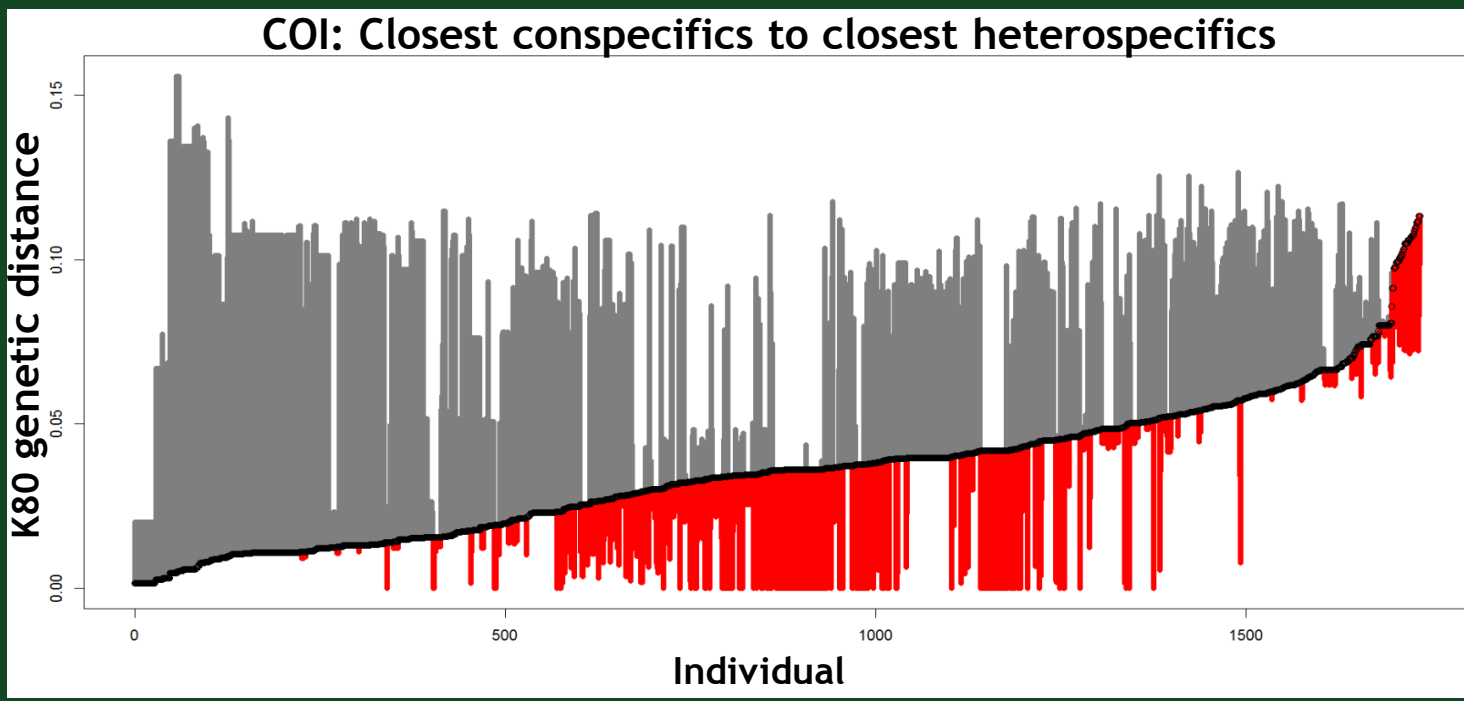

6 EMS of main focus
Aedes aegypti ©ecdc.europa.eu
Aedes albopictus ©ecdc.europa.eu
Aedes japonicus ©ecdc.europa.eu
Aedes koreicus ©ecdc.europa.eu
Aedes atropalpus ©ecdc.europa.eu
Aedes triseriatus ©ecdc.europa.eu

UP: NJ-tree inferred by MEGA. Bootstrap values are shown at the branches;

RIGHT ➜ : Barcoding gap analysis realized on a subset of 1739 COI barcodes (658 bp) with the R package SPIDER;

⬅ LEFT: Elaborated workflow for species identifications of mosquito samples collected in Belgium.

COI: Closest conspecifics to closest heterospecifics

DNA workflow for Belgian mosquito species identification

DNA extractions and eventually quantification → PCR with LCO1490/HCO2198 primers (COI) → If failure: PCR with C1N2181/C1J1718 primers (COI) → Sequencing and species identification analyses

Further tests needed- in progress

IF species identified as:
- Aedes communis
- Aedes detritus
- Aedes flavescens
- Aedes geniculatus
- Aedes punctor
- Aedes rusticus
- Aedes sticticus
- Aedes vexans
- Anopheles claviger
- Anopheles plumbeus
- Culex hortensis
- Culex modestus
- Culex territans
- Culiseta annulata
- Culiseta fumipennis
- Culiseta morsitans
- Culiseta ochroptera
- Coquillettidia richiardii
- Aedes aegypti
- Aedes albopictus
- Aedes atropalpus
- Aedes triseriatus
- Aedes japonicus
- Aedes koreicus

Reliable ID: STOP

IF species identified as:
- Anopheles atroparvus
- Anopheles maculipennis
- Anopheles messeae

PCR with 28SR/5,8SF primers (ITS2) → Sequencing and species identification analyses → Reliable ID: STOP

IF species identified as:
- Culex quinquefasciatus
- Culex pipiens
- Culex torrentium

PCR with ACEquin/ACEtorr/ ACEpip and B1246s primers (ACE2) → Fragment size analysis run on agarose gel

IF species identified as:
- Cx. quinquefasciatus
- Cx. torrentium
Reliable ID: STOP

IF species identified as:
- Cx. pipiens

PCR with pipCQ11R/molCQ11R and CQ11F2 primers (CQ11) and run on agarose gel

IF biotypes identified as:
- Cx. p. biotype molestus
- Cx. p. biotype pipiens
Reliable ID: STOP

IF species identified as:
- Aedes annulipes
- Aedes cantans
- Aedes caspius
- Aedes dorsalis
- Aedes cinereus
- Aedes geminus
- Culiseta subochrea

All native Belgian mosquito species (except seven), and the six investigated exotic mosquito species can be reliably identified using DNA-based methods, based upon data analyses of online repositories and subsequent testing. Further specific identification techniques for the seven remaining native species are currently investigated and/or developed in order to complete the identification workflow. Early interceptions of EMS and a rapid DNA-based identification are thus feasible and will help the authorities in their decision process. Ultimately, the project will build a DNA-reference collection of all life stages of Belgian native and exotic mosquito species.

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