A PIPELINE FOR DNA-BASED IDENTIFICATION OF EXOTIC AND NATIVE MOSQUITOES IN BELGIUM

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BopCo

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AIM
Aedes Invasive Mosquitoes

cost
EUROPEAN COOPERATION
IN SCIENCE & TECHNOLOGY

2nd ANNUAL CONFERENCE

12-13 FEBRUARY 2020, LISBON, PORTUGAL

BOPCO@NATURALSCIENCES.BE HTTP://BOPCO.MYSPECIES.INFO/

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HEALTH & ENVIRONMENT
museum
LifeWatch

BopCo: A Barcoding Facility for Organisms and Tissues of Policy Concern

- Policy concern organisms = endangered species, invasive alien species, human and veterinary disease organisms or their vectors, species of forensic relevance, agricultural pest species, organisms of the human food chain, etc.
- For all stakeholders, using ‘traditional’ morphological characteristics and/or DNA-based identification technology

1. BACKGROUND

BopCo

MEMO: Monitoring of Exotic mosquito species

MEMO

INSTITUTE OF TROPICAL MEDICINE ANTWERP

- ➔ Coordinated by the Institute of Tropical Medicine (Belgium);
- ➔ Nationwide monitoring project;
- ➔ Funded by the Belgian federal authorities;
- ➔ 3 years: July 2017- June 2020;
- ➔ Aim: detecting and evaluating the occurrence of exotic mosquito species (EMS), as they represent an increasing threat to human and animal health in temperate regions (main target: *Aedes*).

1. BACKGROUND

MEMO: Monitoring of Exotic mosquito species

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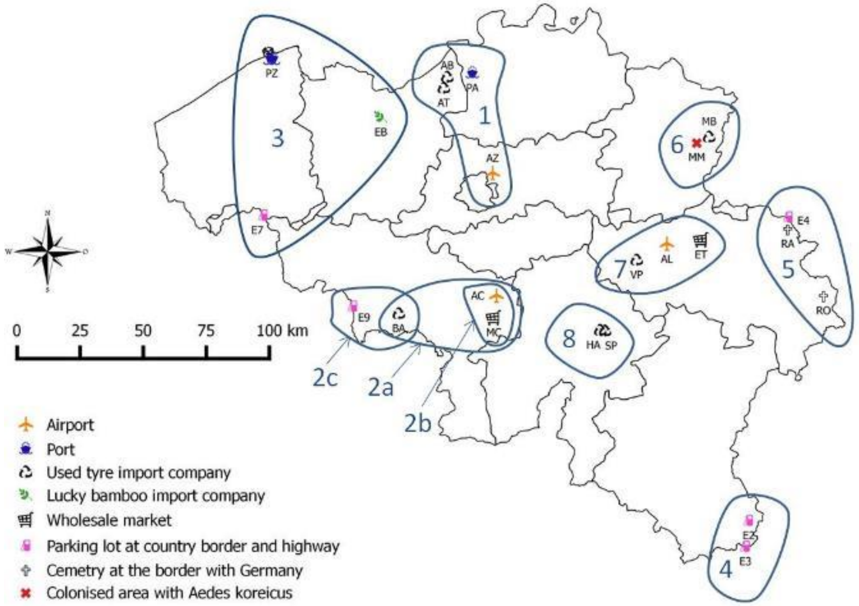
➔ Aim: detecting and evaluating the occurrence of exotic mosquito species (EMS), as they represent an increasing threat to human and animal health in temperate regions (main target: *Aedes*).

➔ **Critical step = Correct species identification**

I. BACKGROUND

Field work: 23 PoEs



-Eggs, larvae and adults collected using 14 Mosquito Magnet Independence Traps, 320 Oviposition Traps, 9 BG-Sentinel Traps.

-146 potential breeding sites are investigated for larvae.

Known introduction pathways: lucky bamboo and used tyre import companies, port and airport, wholesale markets, natural spread.

1. BACKGROUND

Morphological species identification

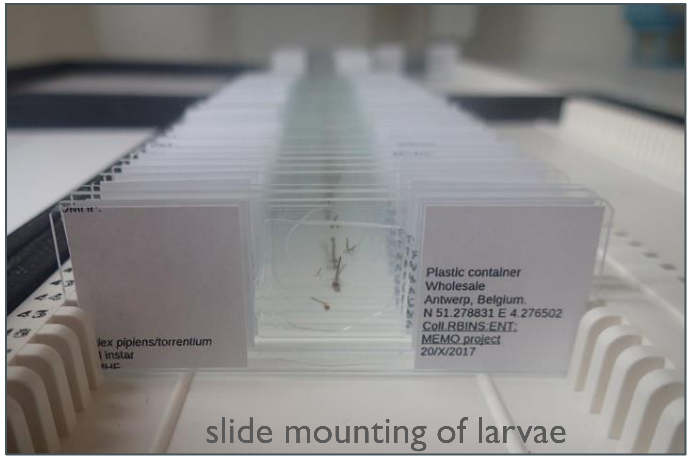
Specimens morphologically identified using:

- Digital identification key of Schaffner *et al.* (2001);
- Printed keys of Schaffner (1993) and Becker *et al.* (2010).

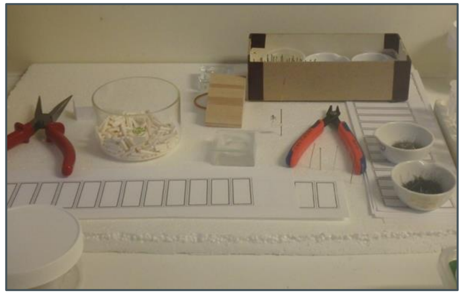
I. BACKGROUND

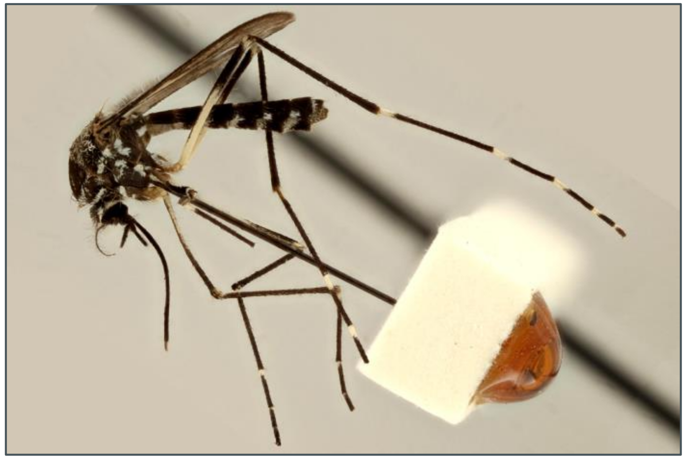
Voucher collection

➔ A few well preserved representatives per species selected: larvae on slides, adults pinned.



slide mounting of larvae





Royal Belgian Insitute for Natural Sciences

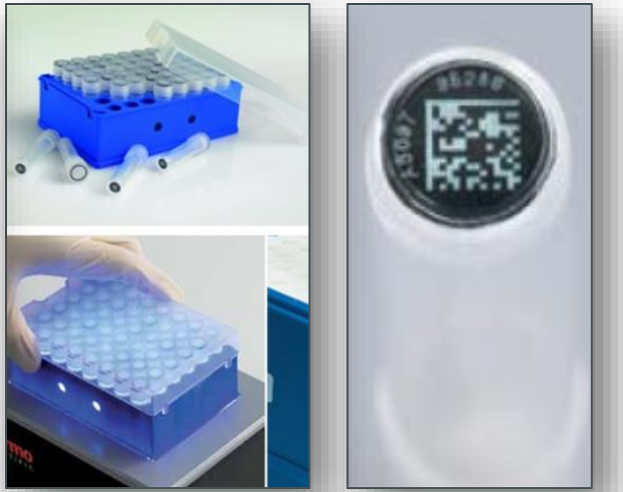
museum

I. BACKGROUND

DNA-based species identification

- **Ad hoc validation** of all (suspected) EMS and all specimens lacking discriminative characteristics (ex. early life stages, damaged or sp complex), mainly focusing on (native) *Aedes*;

- **Annual validation:** random selection of 5% of all caught specimens (adults and larvae) as quality control of the morphological species identifications.



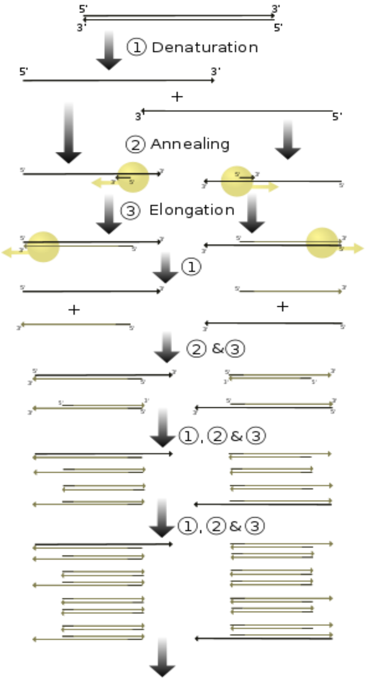
Gentegra techology for tissue and DNA storage

I. BACKGROUND: DNA BARCODING

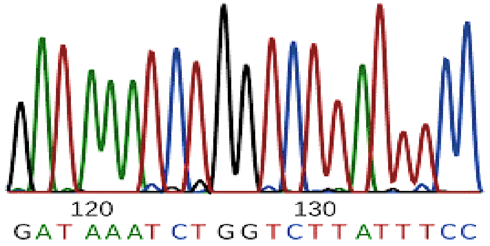
Samples

DNA extraction and quantification

Amplification of selected fragments or loci
(e.g. COI, NADH, ITS2, etc.)

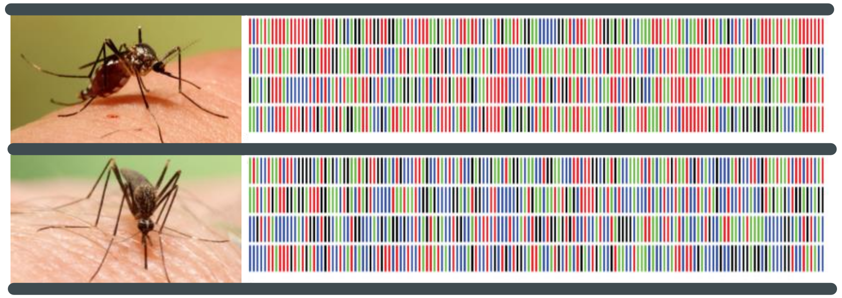


Exponential growth of short product



Sequencing Amplicon

Online repositories database search



√ Species identification

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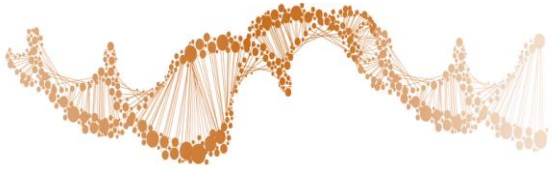
Samples

DNA extraction and quantification



Amplification of selected fragments or loci
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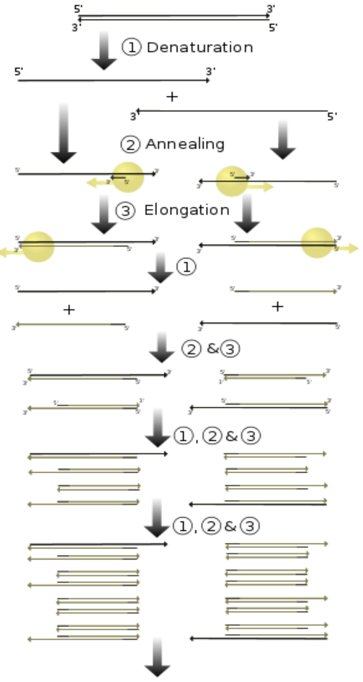
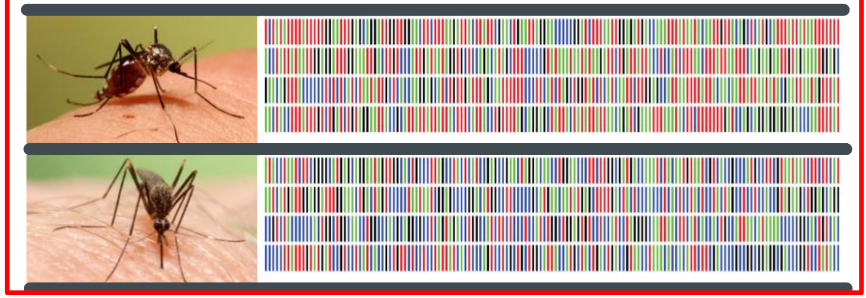


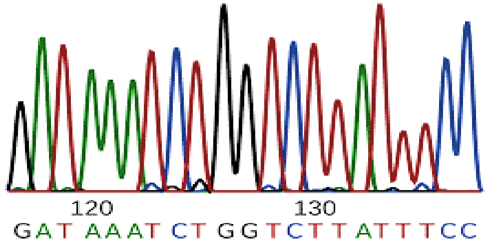
Exponential growth of short product

??? COMPLETENESS AND RELIABILITY ???

√ **Species identification**

Online repositories database search





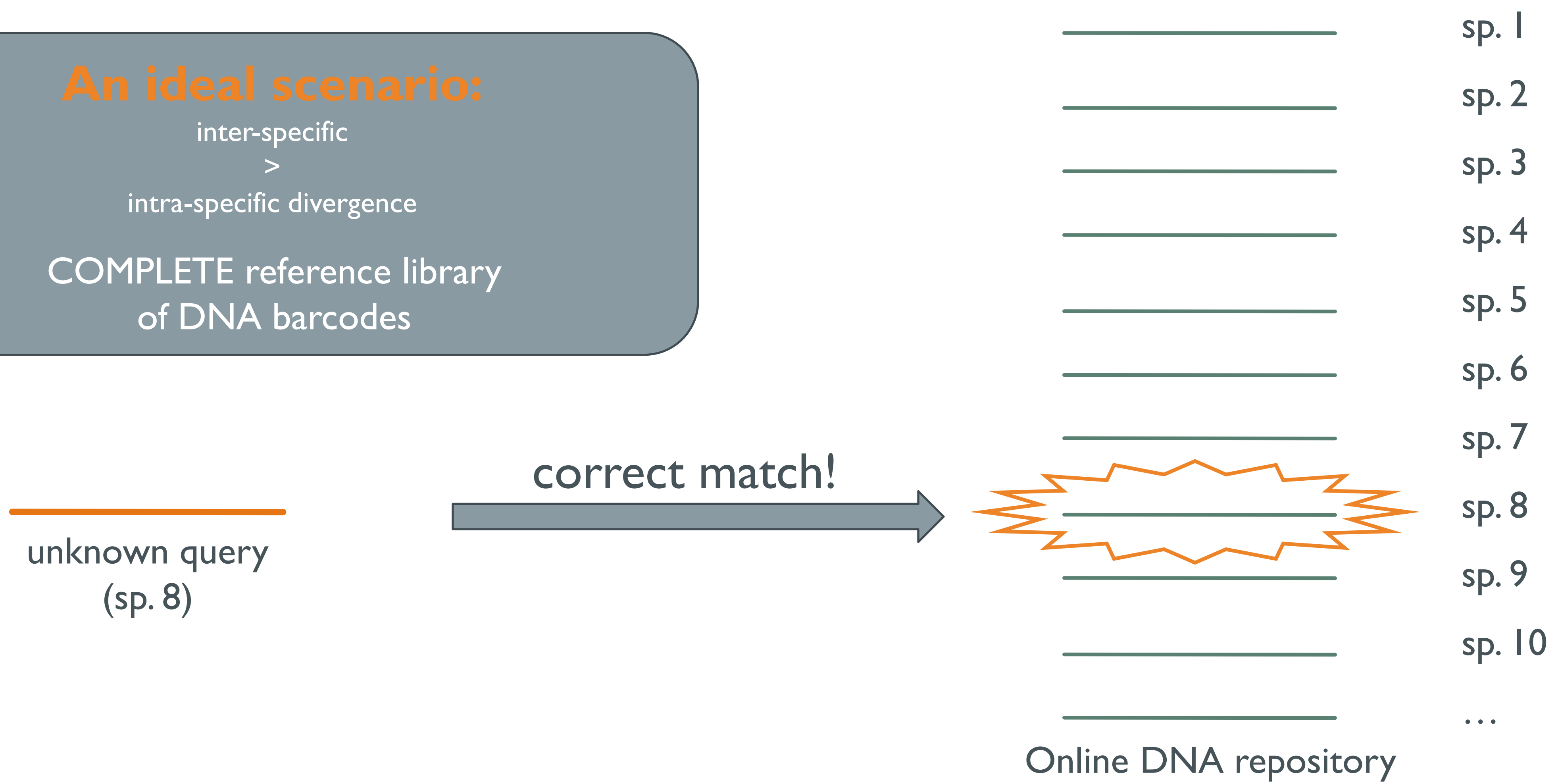
Sequencing Amplicon

1. BACKGROUND: DNA BARCODING

An ideal scenario:

inter-specific
>
intra-specific divergence

COMPLETE reference library
of DNA barcodes



correct match!

unknown query
(sp. 8)

sp. 1
sp. 2
sp. 3
sp. 4
sp. 5
sp. 6
sp. 7
sp. 8
sp. 9
sp. 10
…

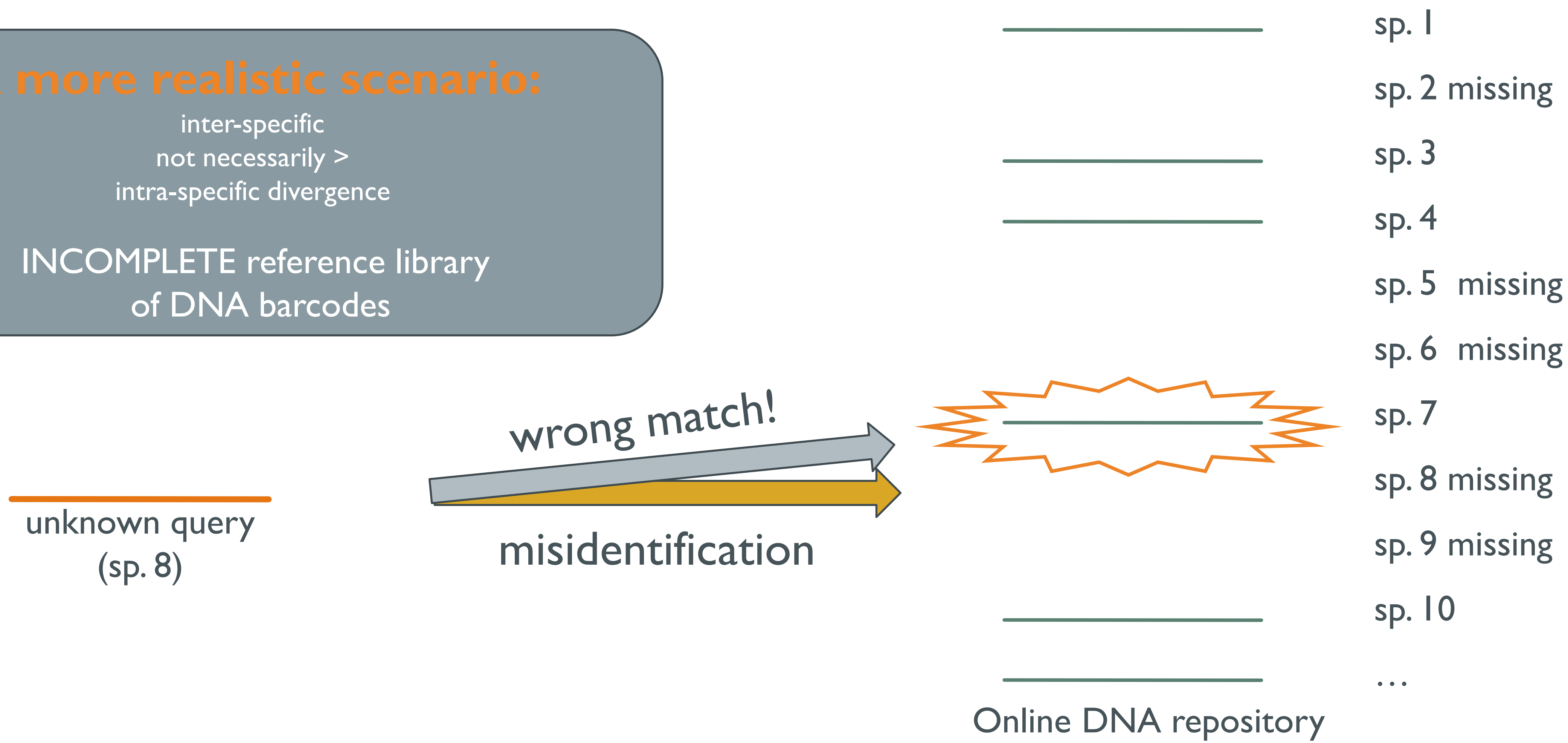
Online DNA repository

I. BACKGROUND: DNA BARCODING

A more realistic scenario:

inter-specific
not necessarily >
intra-specific divergence

INCOMPLETE reference library
of DNA barcodes



sp. 1

sp. 2 missing

sp. 3

sp. 4

sp. 5 missing

sp. 6 missing

sp. 7

sp. 8 missing

sp. 9 missing

sp. 10

…

unknown query
(sp. 8)

wrong match!

misidentification

Online DNA repository

2. METHODS: MOLECULAR SPECIES IDENTIFICATION WORKFLOW

→ EVALUATE THE AVAILABLE REFERENCE LIBRARY OF DNA BARCODES (BOLD & GenBank)

MOLECULAR SPECIES IDENTIFICATION WORKFLOW

➔ EVALUATE THE AVAILABLE REFERENCE LIBRARY OF DNA BARCODES (BOLD & GenBank)

- **Mosquito checklist:** literature review of culicid fauna of Belgium + 7 EMS of main concern to Central Europe + updates from new records for Belgium (3 native and 1 EMS);

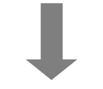
Genus	Species	Belgium →2020
Anopheles	*daciae*	√
	atroparvus	√
	claviger	√
	maculipennis	√
	messeae	√
	pharoensis	√
	plumbeus	√
Aedes	*aegypti*	
	triseriatus	
	cinereus	√
	vexans	√
	geniculatus	√
	atropalpus	
	japonicus	√
	koreicus	√
	annulipes	√
	cantans	√
	caspius	√
	communis	√
	detritus	√
	dorsalis	√
	flavescens	√
	punctor	√
	riparius	
	sticticus	√
	rusticus	√
	albopictus	√
Culex	*pipiens*	√
	torrentium	√
	quinquefasciatus	
	modestus	√
	territans	√
	hortensis	√
Culiseta	*fumipennis*	√
	morsitans	√
	longiareolata	√
	annulata	√
	subochrea	√
Coquillettidia	*richiardii*	√

5 genera; 31 native species;
8 EMS of which 4 recorded in Belgium before 2020

2. METHODS: MOLECULAR SPECIES IDENTIFICATION WORKFLOW

BOLD SYSTEMS

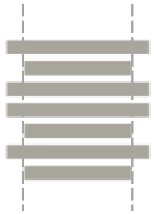
NCBI BLAST



Download all sequence data available

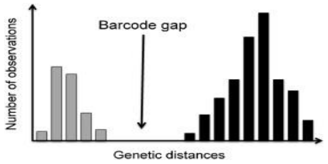
geneious

Filtering the data

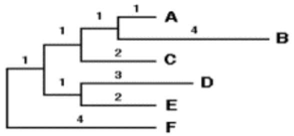


Aligning and trimming of the sequences

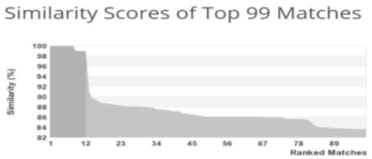
Database



Genetic distance (K2P-distance, p-distance)



Clustering using NJ-tree with Bootstrap support



BLAST-IDS: Basic Local Alignment Tool (BLAST) in GenBank / BOLD

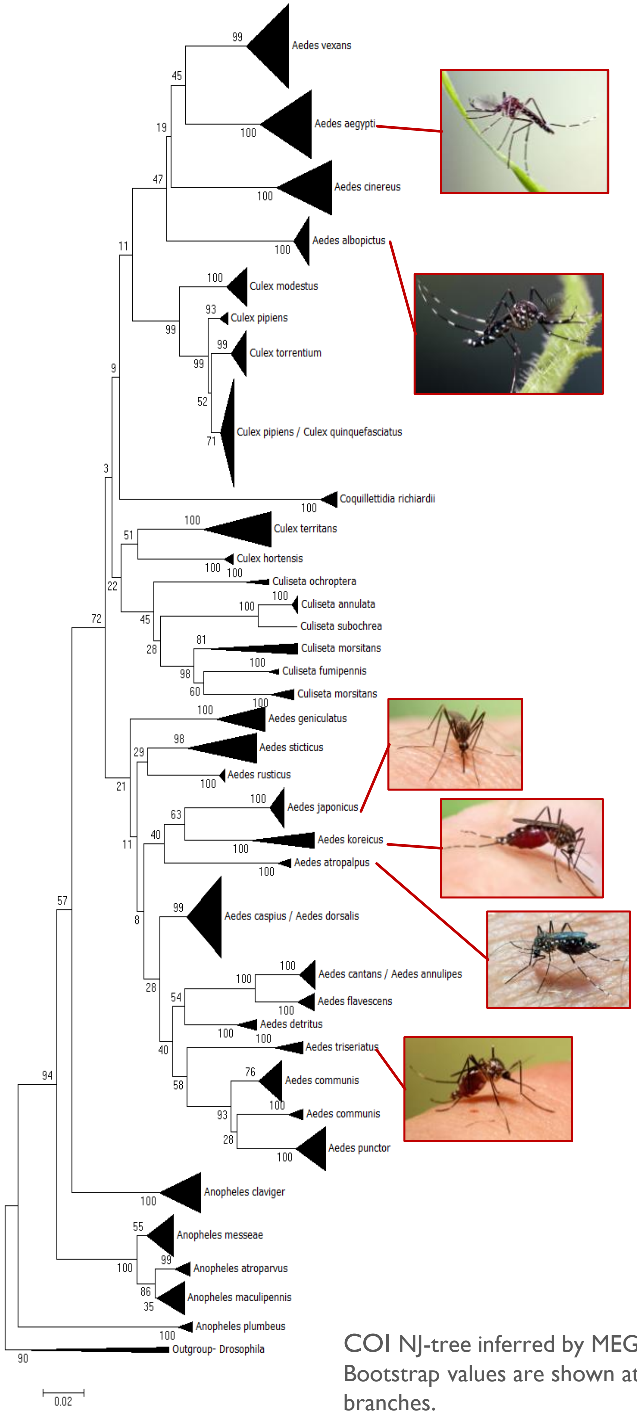
Include metadata like geographical origin of the sequenced voucher/specimen

2. METHODS:

NJ-trees and barcoding gap analyses:

→26 of 39 culicid species identifiable using COI exclusively (including 7 EMS of the checklist);

→8 other species identifiable using other DNA markers (ITS2, ACE2, CQ11 – including 1 EMS of the checklist);

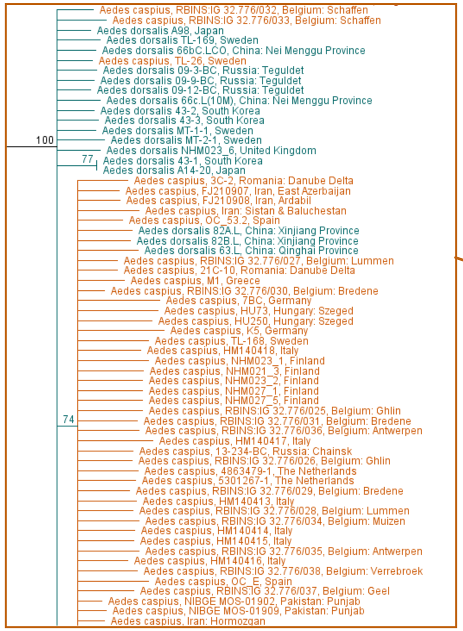


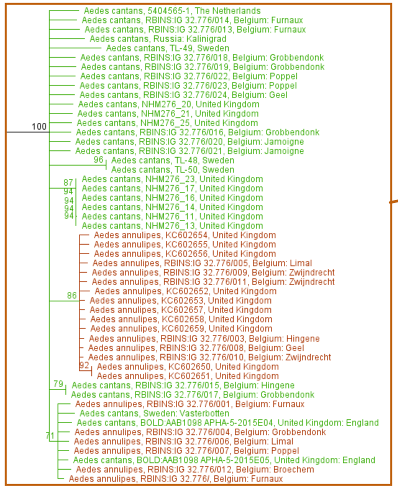
COI NJ-tree inferred by MEGA. Bootstrap values are shown at the branches.

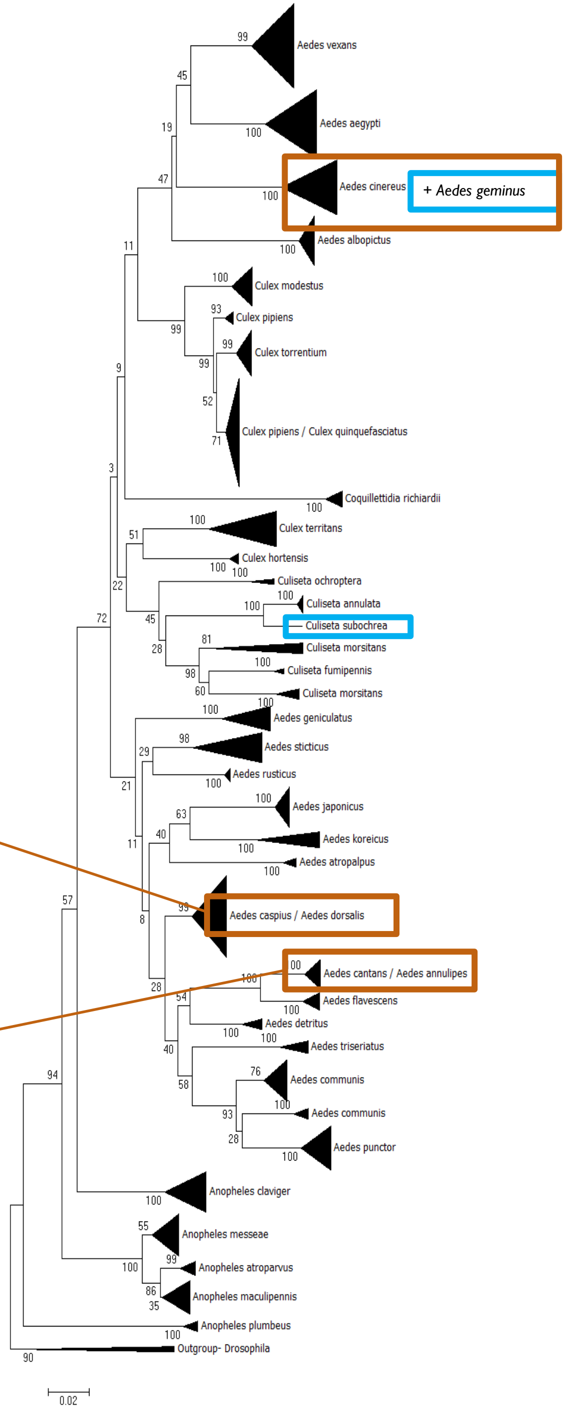
2. METHODS:

→ 3 species complexes (*Aedes caspius / Ae. dorsalis, Ae. cantans / Ae. annulipes, Ae. cinereus / Ae. geminus*)

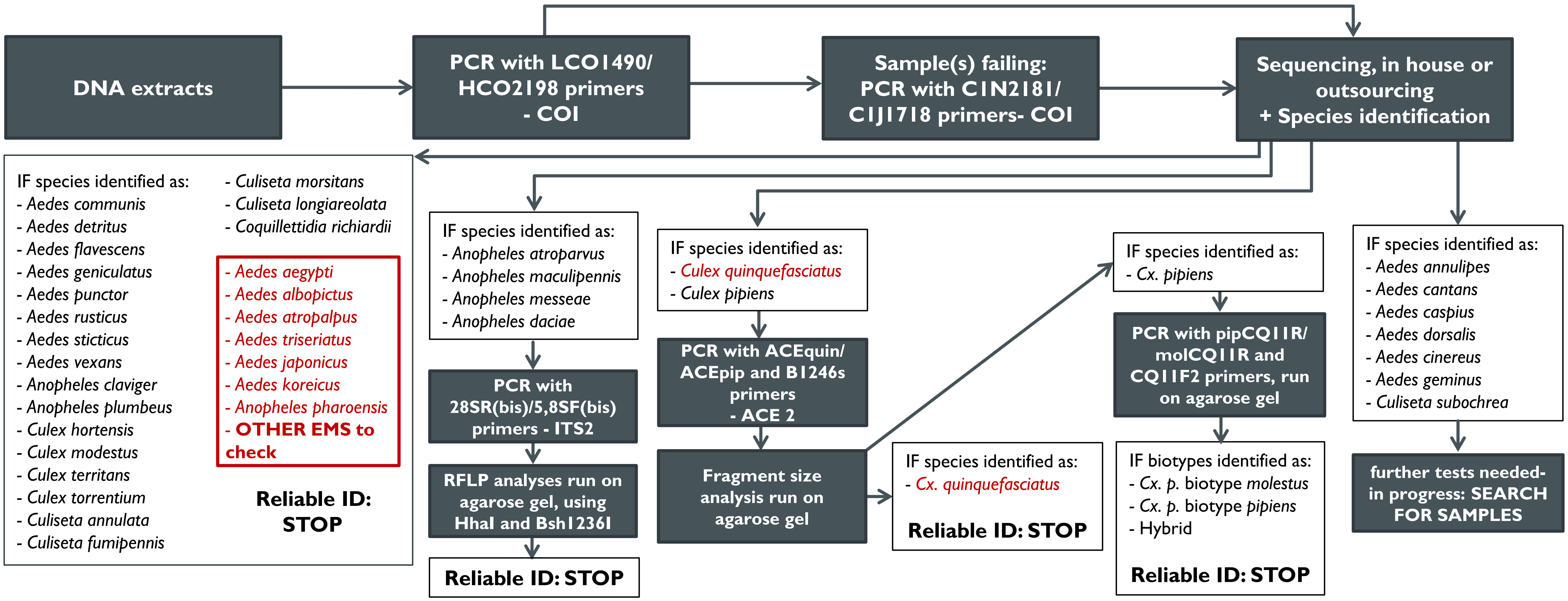
→ 2 species lacking DNA data (*Aedes geminus & Culiseta subochrea*).







2. METHODS: MOLECULAR SPECIES IDENTIFICATION WORKFLOW



DNA extracts

PCR with LCO1490/ HCO2198 primers - COI

Sample(s) failing: PCR with C1N2181/ C1J1718 primers- COI

Sequencing, in house or outsourcing + Species identification

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*
- *Culex torrentium*
- *Culiseta annulata*
- *Culiseta fumipennis*
- *Culiseta morsitans*
- *Culiseta longiareolata*
- *Coquillettidia richiardii*

- *Aedes aegypti*
- *Aedes albopictus*
- *Aedes atropalpus*
- *Aedes triseriatus*
- *Aedes japonicus*
- *Aedes koreicus*
- *Anopheles pharoensis*
- **OTHER EMS to check**

Reliable ID: STOP

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

PCR with 28SR(bis)/5,8SF(bis) primers - ITS2

RFLP analyses run on agarose gel, using HhaI and Bsh1236I

Reliable ID: STOP

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

PCR with ACEquin/ ACEpip and B1246s primers - ACE 2

Fragment size analysis run on agarose gel

IF species identified as:
- *Cx. quinquefasciatus*

Reliable ID: STOP

IF species identified as:
- *Cx. pipiens*

PCR with pipCQ11R/ molCQ11R and CQ11F2 primers, run on agarose gel

IF biotypes identified as:
- *Cx. p.* biotype *molestus*
- *Cx. p.* biotype *pipiens*
- Hybrid

Reliable ID: STOP

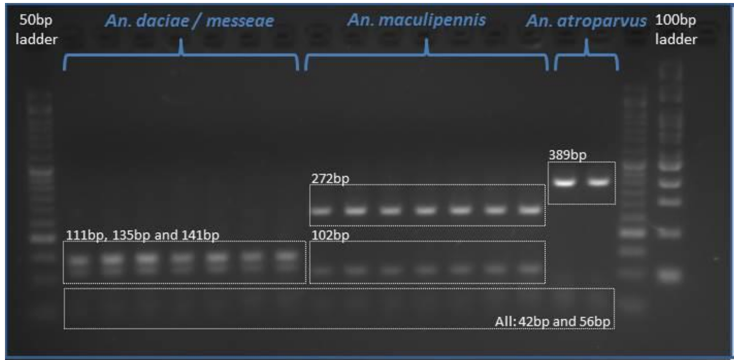
IF species identified as:
- *Aedes annulipes*
- *Aedes cantans*
- *Aedes caspius*
- *Aedes dorsalis*
- *Aedes cinereus*
- *Aedes geminus*
- *Culiseta subochrea*

further tests needed- in progress: SEARCH FOR SAMPLES

2. METHODS: MOLECULAR SPECIES IDENTIFICATION WORKFLOW

Size fragment analyses

➔ *Anopheles maculipennis s.l.*

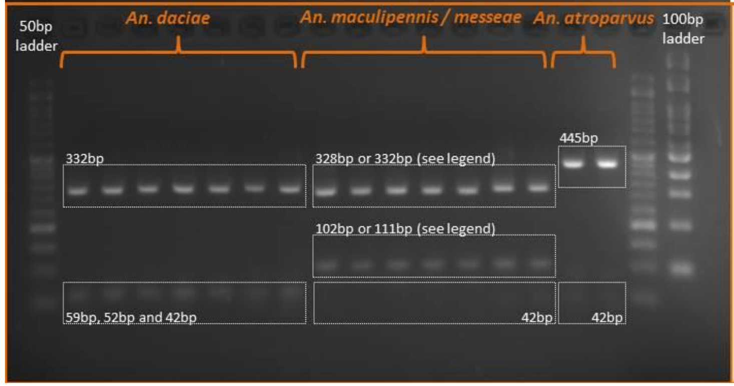


FastDigest HhaI

5'…GCG↓C…3'
3'…C↑GCG…5'

RFLP discriminative technique described in Nicolescu *et al.* 2004

An. messeae and *An. daciae*: 141bp, 135bp, 111bp, 56bp, 42bp



FastDigest Bsh1236I

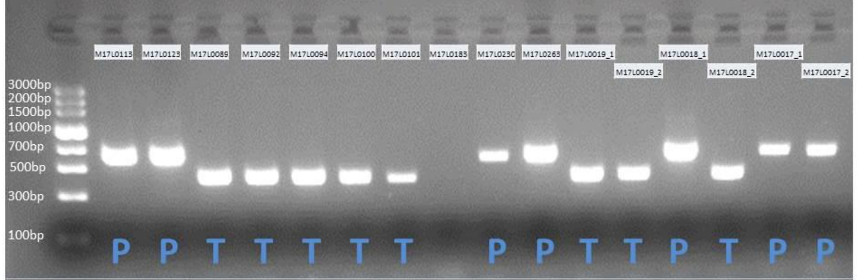
5'…CG↓CG…3'
3'…GC↑GC…5'

RFLP discriminative technique described in Danabalan *et al.* 2014

An. messeae: 332bp, 111bp, 42bp
An. maculipennis: 328bp, 102bp, 42bp

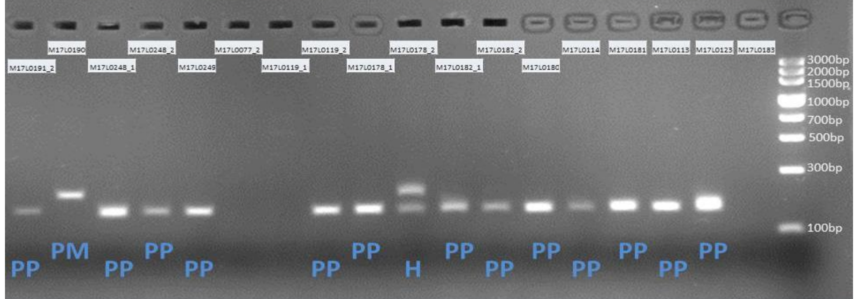
➔ *Culex pipiens s.l.*, *Cx. torrentium* and biotypes

PCR multiplex with B1246s (Forward), ACEpip (Rev), ACEtorr (Rev) and ACEquin (Rev)



P = *Cx. pipiens* (610 bp); T = *Cx. torrentium* (416 bp); Q = *Cx. quinquefasciatus* (274 bp)

PCR multiplex with CQ11F2 (Forward), molCQ11R (Reverse) and pipCQ11R (Reverse)



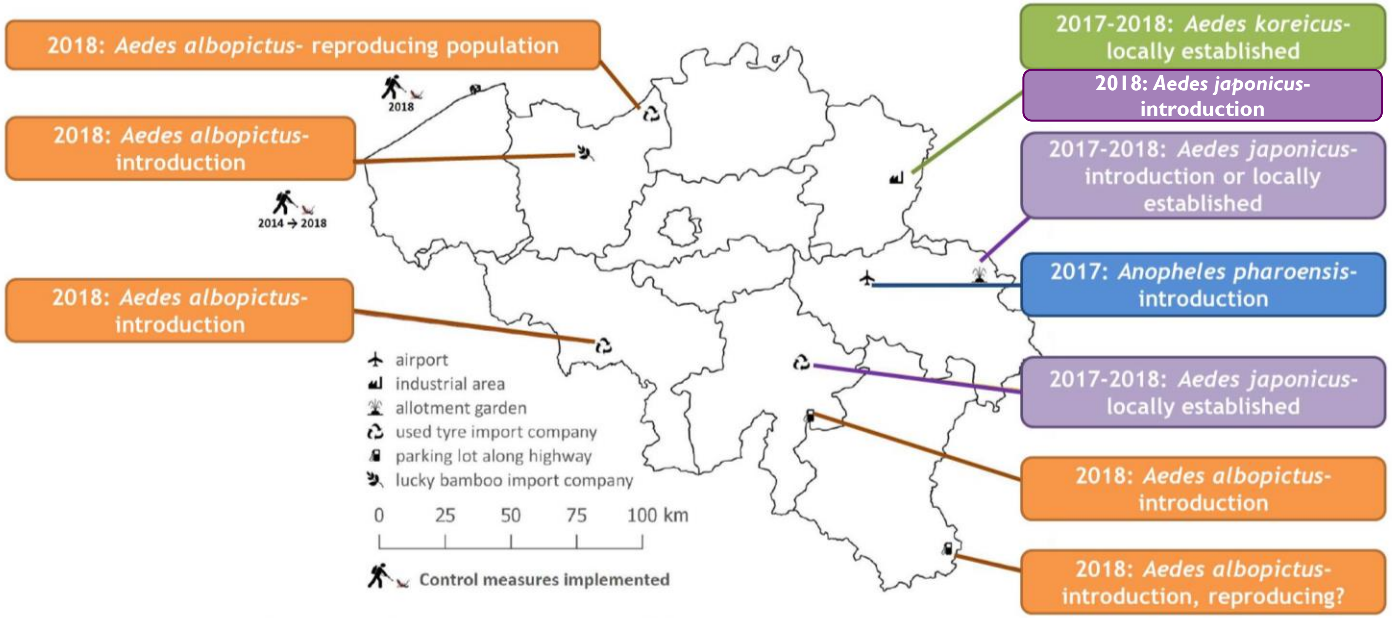
PP = *Culex p.* f. *pipiens* (200 bp); PM = *Culex p.* f. *molestus* (250 bp);
H = Hybrid between biotypes

Alternative qPCR techniques optimized and tested (on progress)

3. RESULTS

Exotic mosquito species occurrence

Four EMS collected once or multiple times at one or multiple PoEs



2018: *Aedes albopictus*- reproducing population

2018: *Aedes albopictus*- introduction

2018: *Aedes albopictus*- introduction

2017-2018: *Aedes koreicus*- locally established

2018: *Aedes japonicus*- introduction

2017-2018: *Aedes japonicus*- introduction or locally established

2017: *Anopheles pharoensis*- introduction

2017-2018: *Aedes japonicus*- locally established

2018: *Aedes albopictus*- introduction

2018: *Aedes albopictus*- introduction, reproducing?

→ Investigate introduction pathway through questionnaires.

→ Investigate possible intensification of the sampling in the surrounding area: established?

→ Investigate possible population genetic study(ies): source?

3. RESULTS

Ex. Annual validation (year 2018)

N = 1,285

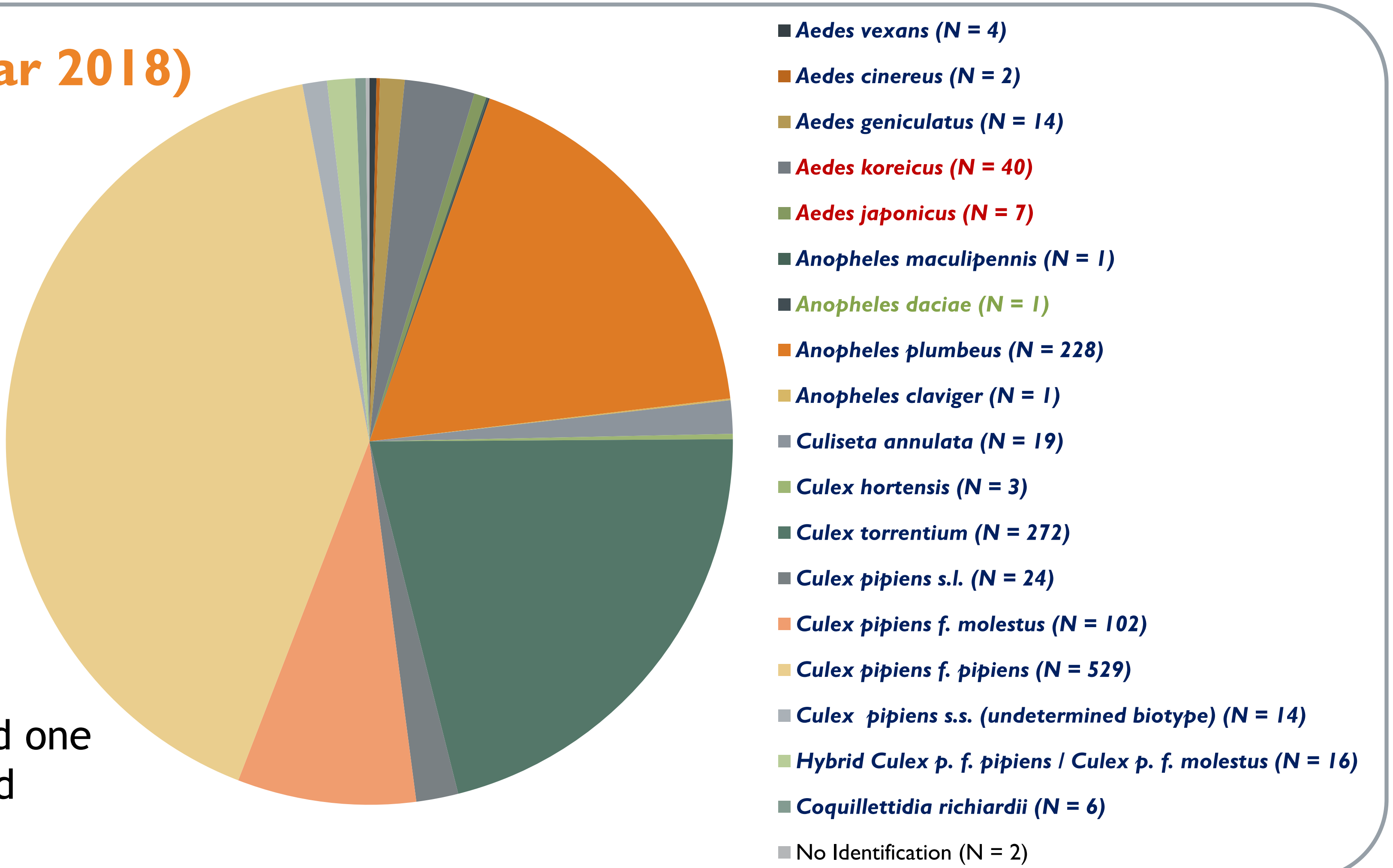
5 genera

→ Most frequent species:

- *Culex pipiens*
- *Anopheles plumbeus*
- *Culex torrentium*

→ Most frequent biotype:

- *Cx. p.* form *pipiens*



➔ Eleven **native**, two **exotic** and one **newly** recorded species validated

4. DISCUSSION

- **Annual validation:**
  - DNA-based identifications allow to validate the morphology-based species identifications (limits for 3 native sp complexes). Yet, it also allows to discriminate between cryptic species, or species of the same complex (*Cx. pipiens s.l. / Cx. torrentium; Anopheles maculipennis s.l.*).

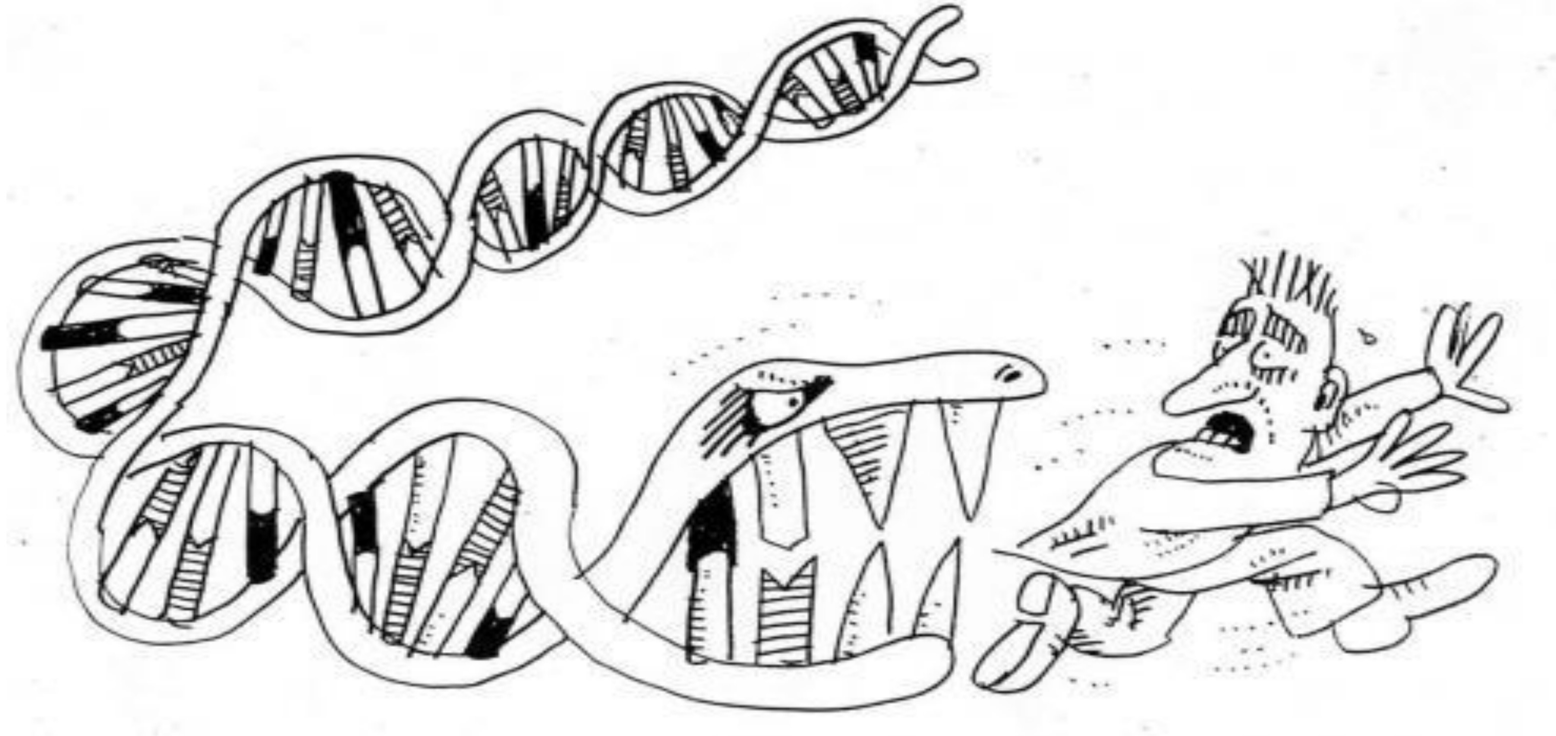
    Every year, more than 98% of the DNA-based identifications matched the respective morphological identifications,

  - DNA-based techniques allow to validate/discover three new species records for Belgium: *Culiseta longiareolata, Culex modestus* and *Anopheles daciae*.

- **Ad hoc validation:**
  - 4 EMS collected at 9 distinct PoEs (2017-2018)
  - Very useful in case of eggs (mainly with *Ae. albopictus* and *Ae. geniculatus*).

➔ EMS seem to enter effectively via different introduction pathways.
Early interceptions and rapid DNA-based verifications should help the authorities in their efforts to contain the spread and eradicate EMS (populations).

QUESTIONS?



BOPCO CONTACT DETAILS

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A Barcoding Facility for Organisms and Tissues of Policy Concern

Welcome to BopCo

The **Barcoding Facility for Organisms and Tissues of Policy Concern** (BopCo) aims at developing an expertise forum to facilitate the identification of biological samples of policy concern. Such identifications can rely on traditional morphology-based approaches requiring taxonomic expertise and/or DNA-based techniques demanding specific skills and access to a fully equipped molecular laboratory.

The intent of BopCo therefore is **(1)** to act as a focal point for identifying biological materials upon request, using both morphological and DNA-based techniques, **(2)** to produce well-documented DNA barcodes of relevant taxa, **(3)** to maintain reference collections of barcoded organisms and the corresponding DNA barcode databases, and **(4)** to explore and implement new tools and techniques for species identification and DNA barcoding.

bopco@naturalsciences.be http://bopco.myspecies.info/