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Molecular confirmation of *Anopheles melas* (Diptera: Culicidae) in Democratic Republic of Congo

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Anopheles melas Theobald had been reported from coastal areas of the Democratic Republic of Congo (DRC), but molecular methods had never previously been used to confirm this identification. To see if *An. melas* was indeed present in the coastal area of DRC, *Anopheles* mosquitoes were collected in larval collections. Those morphologically identified as belonging to the *Anopheles gambiae* complex as adults were identified to species using polymerase chain reaction methods. The identity of those found to be *Anopheles melas* were confirmed through sequencing of the DNA. As *Anopheles melas* has been found to be a competent malaria vector elsewhere (Akogbeto & Romano 1999), these mosquitoes and their habitat should be considered in malaria control activities.

The *Anopheles gambiae* complex (Diptera: Culicidae) is important in malaria transmission in the DRC (Wat'senga *et al*. 2018). The complex is currently composed of nine members: *An. gambiae s.s.*, *An. coluzzii*, *An. arabiensis*, *An. quadriannulatus*, *An. amharicus*, *An. bwambae*, *An. merus*, *An. melas* Theobald, and the recently described *An. fontenillei* (Barrón *et al*. 2019). *Anopheles coluzzii* Coetzee & Wilkerson and *An. gambiae s.s.* Giles have wide-spread distribution in DRC (Bobanga *et al*. 2016). *Anopheles arabiensis* Patton has also been found in eastern DRC (Bandibabone *et al*. 2018). Of the other species in the complex, only *An. melas* Theobald has been reported in the Democratic Republic of Congo (Rahm 1966), on the basis that the *An. gambiae* collected by Wanson (1935a, b) from brackish water in crab holes in Banana must be *Anopheles melas*. These specimens were collected at a time when molecular methods for species identification were not available and, according to De Meillon (1947), the characteristics used to distinguish *An. melas* from *An. gambiae* were later determined to be inadequate. The aim of this study was therefore to use modern molecular methods to confirm that *An. melas* was present in the short Atlantic coastal area of Kongo Central.

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Mosquitoes were collected in Banana, DRC (−−5.997462, 12.394224) (Fig. 1). Banana is a small village on a spit of land where the Congo River flows into the Atlantic Ocean. The village has sandy soil with swamps nearby, and the village is about 8 km south of Muanda. Banana has two main seasons: a dry season that lasts from May to October, and a rainy season that lasts from October to May.

Larval collection was the method used to find mosquitoes in this study (WHO 2013). Larvae were taken from brackish water in crab holes using ladles and pipettes. Larvae were returned to the laboratory and reared to the adult stage before identification of species.

Mosquitoes collected were identified to species using the keys of Gillies & De Meillon (1968), and Gillies & Coetzee (1987). All mosquitoes that were identified as *Anopheles gambiae s.l.* were kept in 1.5-ml tubes with silica gel for preservation. These were sent to the Entomology Branch at the Centers for Disease Control and Prevention (Atlanta, GA, U.S.A.) for analysis.

DNA was extracted from the mosquitoes using Extracta DNA Prep (Quanta Biosciences, Gaithersburg, MD, U.S.A.). The extraction protocol was modified to account for the small amount of tissue by using only 25 μl of the Extraction Reagent, and 25 μl of the Stabilization Buffer. PCR was performed using AccuStart II GelTrack PCR Super-Mix (Quanta Biosciences, Gaithersburg, MD, U.S.A.) at three-quarters of the manufacturer's recommended concentration. Primers, concentrations, and conditions were as described in Wilkins *et al.* (2006), a standard protocol used in the CDC laboratory. Three positive controls were included in each PCR, one specific for each of the following species: *Anopheles melas*, *Anopheles gambiae s.s.* and *Anopheles merus*.

Identity of the specimens that tested positive as *Anopheles melas* was confirmed with direct sequencing. Larger volume PCR reactions were performed as above using only primers IMP-UN and ME-3T. The PCR products were purified using the MultiScreen HTS PCR96 Filter Plate (Millipore, Billerica, MA, U.S.A.). One-quarter sequencing reactions were performed with BigDye Terminator v1.1 and purified with the BigDye XTerminator Purification Kit (Life Technologies, Foster City, CA, U.S.A.). The purified sequence reactions were run on a 3500xL Genetic Analyzer (Life Technologies, Foster City, CA, U.S.A.) using the BDx Fast module according to the manufacturer's instructions. The sequence data were analysed using SeqMan Pro from the DNASTAR Lasergene 12 Core Suite (DNASTAR, Madison, WI, U.S.A.). The consensus sequences were compared against known sequences in the Nucleotide database of GenBank using the BLAST tool.

Six of the collected mosquitoes were identified as *Anopheles gambiae s.l.* and were processed in the laboratory. Four specimens tested positive as *An. melas* and had a 100 % match to *Anopheles melas* in GenBank (exact match with AH006429.2). The other specimens (2) were both found to be *Anopheles gambiae s.s.*

It is not surprising that *An. melas* was found in Banana, as the species had been confirmed in Kikudo, Matajor, Angola (6°07'S 12°22'E) in 2002 (Calzetta *et al.* 2008). Similarly, Sinka *et al.* (2010) predicted the presence of *An. melas* along the entire Atlantic coastline of DRC. Nevertheless, the confirmation of the presence of this species in Banana opens doors

to future research, such as the importance of this species in malaria transmission and its resistance to insecticides.

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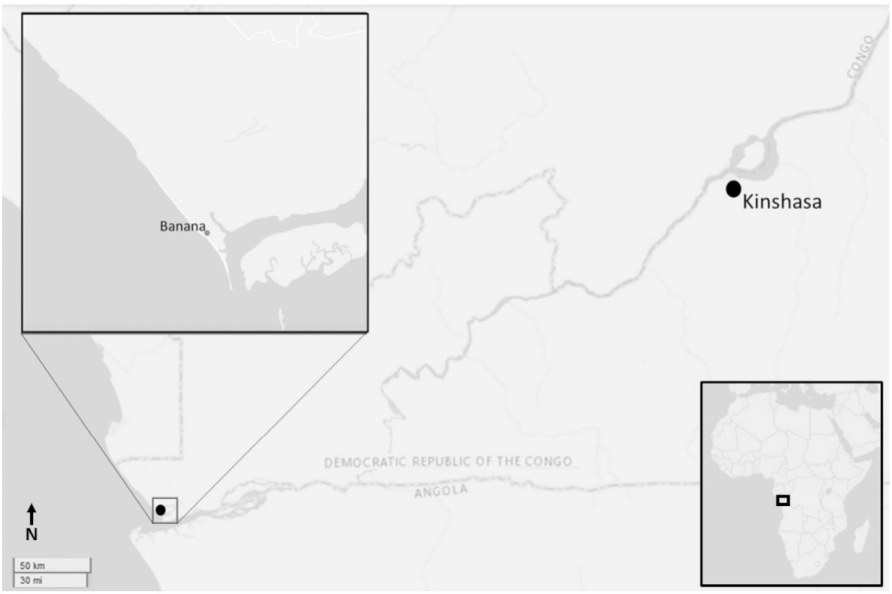

Fig. 1.
Kongo Central, showing the study site (Banana).