

Description and Bionomics of *Anopheles (Cellia) ovengensis* (Diptera: Culicidae), a New Malaria Vector Species of the *Anopheles nili* Group from South Cameroon

H. P. AWONO-AMBENE,^{1, 2} P. KENGNE,³ F. SIMARD,² C. ANTONIO-NKONDJIO,²
AND D. FONTENILLE³

Laboratoire de Recherche sur le Paludisme, OCEAC, P.O. Box 288, Yaoundé, Cameroon

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ABSTRACT Mosquito species of the *Anopheles nili* group (Diptera: Culicidae) transmit malaria to humans along rivers in Africa. To date, the *An. nili* group includes the species *Anopheles nili* s.s. and its pale-winged variant known as the “Congo form,” *Anopheles somalicus* and *Anopheles carnevalei*. Larval and adult mosquito collections in the forest region of Campo, in southern Cameroon, uncovered an additional morphological variant provisionally called “Oveng form” that was subsequently found to be genetically distinct from the other members of the *An. nili* group. In this study, we provide further biological data that characterizes this new taxon and justifies elevation to specific rank. We propose calling this new species *Anopheles ovengensis*, after its geographical origin. We present a morphological description of the adult female and fourth instars and original data on the biology, ecology, and role as a human malaria vector of this new species in its type location. We provide dichotomous keys for identification of adult females and fourth instars that can be used at least in tropical areas of west and central Africa.

KEY WORDS bionomics, *Anopheles ovengensis*, *Anopheles nili* group, Culicidae, Cameroon

MOSQUITOES BELONGING TO THE *Anopheles nili* group are major human malaria vectors in tropical Africa, especially along streams and rivers that represent typical larval development sites (Gillies and De Meillon 1968, Krafur 1970, Carnevale et al. 1992, Antonio-Nkondjio et al. 2002). Based on extensive morphological, ecological, and ethological variations reported by many authors among wild *An. nili* s.l. populations (Gillies and De Meillon 1968, Carnevale et al. 1992, Brunhes et al. 1999), three species and one variation were described. The highly anthropophilic *Anopheles nili* s.s. (Theobald, 1904) extends throughout most of intertropical Africa, from southern Senegal to Sudan and into northern South Africa (Hamon and Mouchet 1961, Gillies and De Meillon 1968). In the Congo basin, a pale-winged variant known as the “Congo form” (De Meillon 1947) has been reported, but no biological and/or genetic data allowed elevation to specific rank. The recently described *Anopheles carnevalei* (Brunhes et al. 1999) differs from *An. nili* s.s. based upon the abundance of clear spots on its wings and has so far been reported only from equatorial forest

regions of Ivory Coast and Cameroon. Finally, the probably widespread but largely unrecognized *An. somalicus* (Rivola and Holstein 1957) is characterized by zoophilic and exophilic feeding habits. Diagnostic morphological characters are observable at the larval and pupal stages only, whereas adult specimens are basically indistinguishable from typical *An. nili* s.s. (Gillies and De Meillon 1968, Gillies and Coetzee 1987). Studies on *An. nili* populations from forest villages Oveng and Nyabessan, near Campo in southern Cameroon, revealed an additional morphological variant provisionally called “Oveng form.” Recently, Kengne et al. (2003) provided full support for splitting *An. nili* s.l. into four taxonomic units, based on segregating sequence differences in the r-DNA ITS2 and D3 domain. All sequences obtained from the Congo form seemed indistinguishable from *An. nili* s.s., whereas fixed differences were indeed observed between *An. nili* s.s., *An. carnevalei*, *An. somalicus*, and Oveng form specimens. Both morphological and genetic data therefore indicated that the Oveng form is a new species within the *An. nili* group. Here, we provide a morphological description of this new species and updated data on its bionomics.

¹ Medical Research Centre, Institute of Medical Research and Medicinal Plant studies, P.O. Box 1457 MINREST, Yaoundé, Cameroon.

² Laboratoire IRD de recherche sur le paludisme, Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale, P.O. Box 288 Yaoundé, Cameroon.

³ Laboratoire de Lutte contre les Insectes Nuisibles, Institut de Recherche pour le Développement, 911 Avenue Agropolis, P.O. Box 64501, 34394 Montpellier Cedex 5, France.

Materials and Methods

Sampling Area

Mosquitoes were collected in Oveng (2° 10' N, 10° 30' E) and Nyabessan (2° 10' N, 10° 40' E). These are

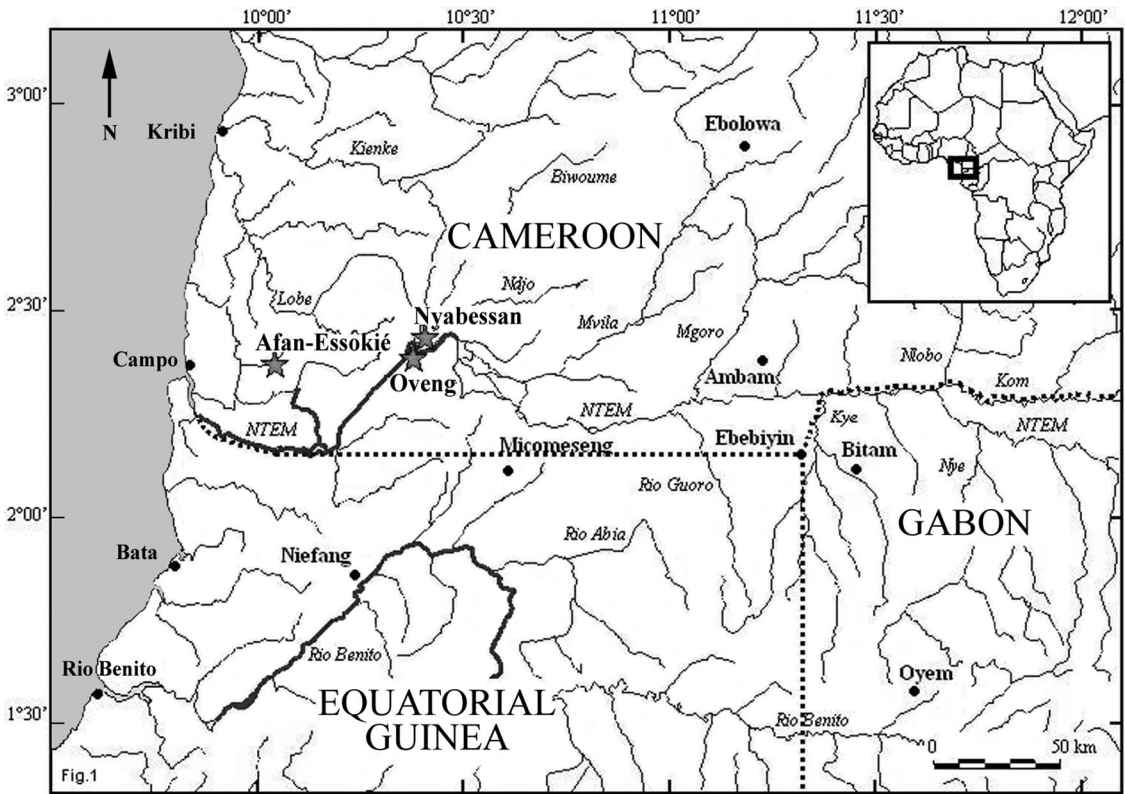


Fig. 1. Map of the study area showing the Ntem River hydrographic network in South Cameroon. Stars denote the type location of *An. ovengensis*: the village of Oveng ($2^{\circ} 10' N$, $10^{\circ} 30' E$), and the neighboring villages Nyabessan ($2^{\circ} 10' N$, $10^{\circ} 40' E$) and Afan-Essokié ($2^{\circ} 20' N$, $10^{\circ} 00' E$) where adults and larvae were sampled. Italics, river names; bold dot, major localities; ■■■ borders. Inset, map of Africa showing the study area.

two neighboring villages (~ 5 km apart) within the forested equatorial domain of southern Cameroon, located on both sides of the Ntem River, near natural waterfalls (Me'nvele). These villages are ≈ 250 km distant from Yaounde, the capital city of Cameroon, and ≈ 400 m above sea level. Annual rainfall typically ranges from 1,700 to 2,500 mm, occurring mostly during two rainy seasons, from April to June and from September to November (Olivry 1986). The hydrographic network centered on the Ntem River also covers northern areas of Gabon and Equatorial Guinea (Fig. 1).

Mosquito Sampling and Taxonomic Procedures

Female mosquitoes were caught at night after landing on human volunteers (indoors and/or outdoors), on the edges of the rivers. Larvae were collected in breeding sites, along rivers crossing the study area. Adult *An. nili s.l.* mosquitoes were morphologically identified to species according to Gillies and De Meillon (1968) and Brunhes et al. (1999). Field-collected larvae of *An. ovengensis* and discarded larval exuviae were collected and stored in 70% ethanol and then mounted individually in Euparal for observation and morphological description. The adults were

mounted on card triangles on insect pins. The wings, legs, and palps were carefully removed from the body and dry mounted on a microscope slide. Cheatotaxy and nomenclature used for material descriptions follow that of Harbach and Knight (1980), Reinert et al. (1997), and Nguyen Duc Manh et al. (2000). The new species is recognized on the basis of correlated anatomical features in associated life stages. Diagnostic and differential characters were confirmed in all available specimens, including five female adults and two fourth instars. No male *An. ovengensis* were collected at the adult stage, and no males were observed among emerging adults, probably because very few specimens reached this stage of development under our insectary conditions.

The holotype and a paratype are deposited in the Laboratoire de Taxonomie des Vecteurs, Institut de Recherche pour le Développement (IRD), Montpellier, France. Three paratypes are also deposited in the British Museum (Natural History), London, United Kingdom; in the Muséum National d'Histoire Naturelle, Paris, France; and in the Laboratoire de Recherche sur le Paludisme, Organisation de Coopération pour la lutte contre les Endémies en Afrique Centrale (OCEAC), Yaoundé, Cameroon.

Table 1. Morphological and biometric diagnostic characters on fourth instars in the *An. nili* group

Morphological feature	<i>An. ovengensis</i>	<i>An. carnevalei</i> (undescribed)	<i>An. somalicus</i> (after Gillies and De Meillon, 1968)	<i>An. nili</i> s.s. (after Gillies and De Meillon, 1968)	Congo form (after Gillies and De Meillon, 1968)
Seta 3-C size (ratio size: 3-C/2-C)	Long (0.7–0.8)	Short (<0.5)	Long (≥1.0)	Long (0.7–0.8)	Long (0.7–0.8)
Seta 3-C aspect	Simple	Apically forked	Simple	Simple	Simple
Seta 1-P	Dendritic	Forked	Dendritic	Simple	Simple
No. of accessory plates on III–VI segments	1	3	1	1	Unknown

Table 2. Morphological and biometric diagnostic characters on wing of adult females in the *An. nili* group

Morphological feature of wings	<i>An. ovengensis</i>	<i>An. carnevalei</i>	<i>An. somalicus</i> (after Rivola and Holstein, 1957)	<i>An. nili</i> s.s. (after Gillies and De Meillon, 1968)	Congo form (after Gillies and De Meillon, 1968)
No. of pale spots on costa and radius vein	4	4	4 (or 3)	3 (or 4)	4
Presector pale spot on radius vein	Large	Large	Narrow	Narrow	Narrow
Median pale scales on R2 and M1 veins	None	Present	None	None	None
No. of pale spots on alar fringe	4	4	3	2	4

Molecular Characterization

DNA was extracted from individual mosquitoes at both adult and larval stages and the ITS2 region was amplified using polymerase chain reaction (PCR) as described in Kengne et al. (2003). Briefly, we used one universal forward primer (ANU) and four reverse primers, ANO, ANC, ANS, and ANT, respectively, specific to *An. ovengensis* n.sp., *An. carnevalei*, *An. somalicus*, and *An. nili*. Sequences were as follows: ANU: 5'GATGCACACATTCTTGAGTGCC3'; ANO: 5'AGCACGGTCACCTACGGTTCTCC3'; ANC: 5'CTGGTGGGGTTCTTCTCTTCTCC3'; ANS: 5'ATGCACCAGGGGTTTGGGCC3'; and ANT: 5'TGGCTGCTTCTCGTGCCGCG3'.

PCR mixture consisted of 1.5 mM MgCl₂, 200 μM each dNTP (Eurogentec, Ougrée, Belgium), 2.5 μl of 10× *Taq* buffer, 0.625 U of *Taq* polymerase (QIAGEN, Courtaboeuf, France), and 10 ng of template DNA in 25 μl final reaction volume. The amount of each primer used in the PCR assay was 40 pmol for ANU and 10 pmol each for ANO, ANC, ANS, and ANT. PCR conditions included an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C with a final extension step of 10 min at 72°C. The amplified fragments were separated by electrophoresis on a 2% agarose gel.

Description of *An. ovengensis*

Diagnosis. Diagnostic characters in the larval stages between *An. ovengensis* and other species of the *An. nili* group are reported in Table 1. The female adult of *An. ovengensis* is morphologically similar to the formerly described members of the *An. nili* group, with an apical white spot on palps and dark legs. It can be distinguished from other members of the *An. nili* group by characters shown in Table 2.

Comparison of sequence polymorphism in different regions of rDNA revealed fixed differences between *An. nili* s.s. (including the Congo form), *An. somalicus*,

and *An. ovengensis*. A diagnostic PCR assay was implemented based on specific sequence differences in the ITS2 region (see Materials and Methods). The size of the diagnostic band revealed on regular agarose gels is 188 bp for *An. nili*, 357 bp for *An. ovengensis*, 408 bp for *An. carnevalei*, and 329 bp for *An. somalicus* (Kengne et al. 2003).

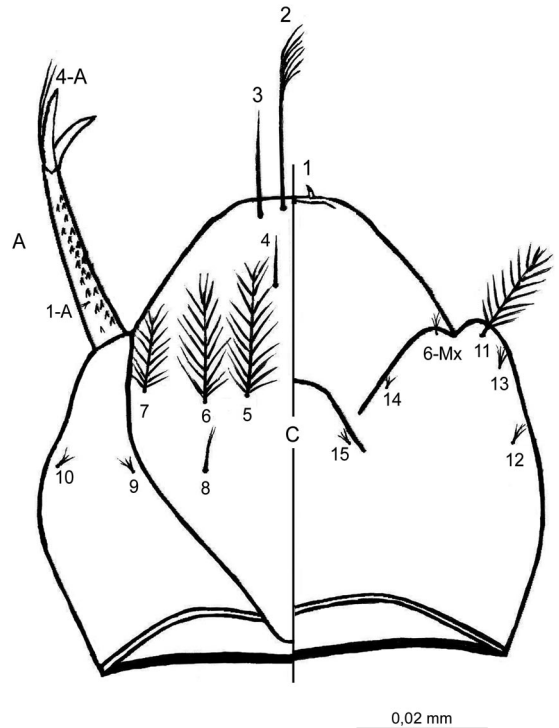


Fig. 2. Fourth-stage larva of *An. ovengensis*. Head, half dorsal view (left) and half ventral view (right) of cranium. A, antenna; C, cranium; 1–15, setal numbers for specified areas, e.g., seta 2-C.

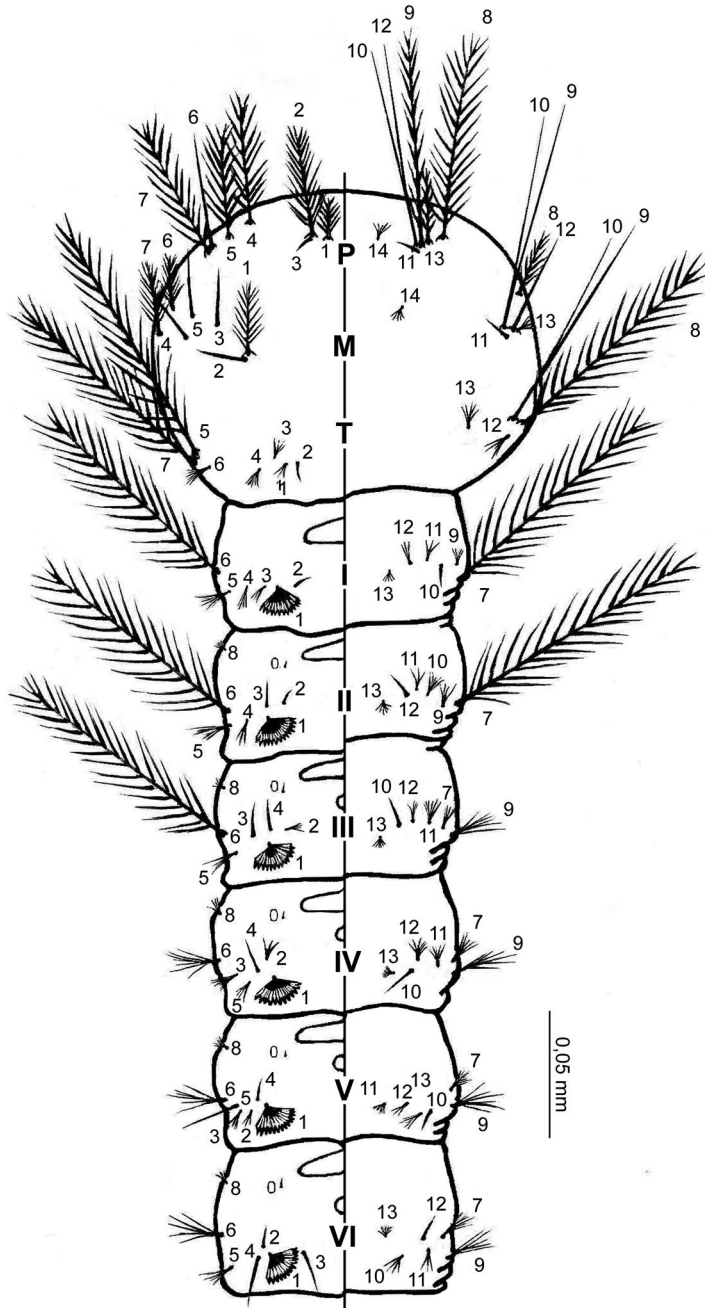


Fig. 3. Fourth-stage larva of *An. ovengensis*. Fourth instar thorax and abdominal segments I-VI (dorsal view on left half and ventral view on right half). P, prothorax; M, mesothorax; T, metathorax; I-VI, abdominal segments I-VI; seta 11-T not shown.

Larva, Fourth Stage. Character and positions of setae are shown in Figs. 2, 3, and 4.

Head (Fig. 2). Unevenly pigmented; slightly wider than long (≈ 0.070 mm in length and 0.075 mm in width). Collar and dorsonotum darkly pigmented. Cranium shows, respectively, four pairs of anterior setae (1-C, 2-C, 3-C, and 4-C), three pairs of median

setae (5-C, 6-C, and 7-C), and one pair of posterior setae (8-C). Setae 1-C located in median labral plate are usually short and simple. Setae 2-C show an apical tuft divided into six or eight branches. Setae 3-C simple, about setae 2-C size; setae 4-C close together behind 2-C setae, simple and short. Setae 5-C are well developed, usually up to the base of 4-C setae. Setae

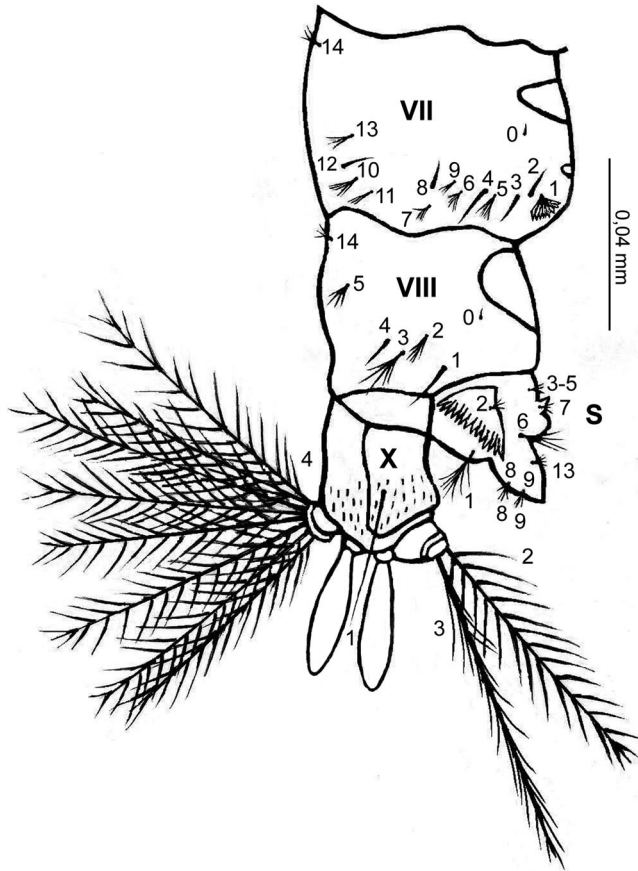


Fig. 4. Fourth-stage larva of *An. ovengensis*. Palmate seta, and "fourth instar lateral" view of abdominal segments VII, VIII, and X. S, siphon.

5-C, 6-C, and 7-C are branched into eight or 10 simple setae on each side. Setae 8-C, short as setae 4-C, simple or divided in two branches. Setae 9-C show three or four branches. Antenna: lightly pigmented, mesal and ventral surfaces strongly spiculated, length ≈ 0.04 mm. Setae 1-A very short, usually with fewer than three branches.

Thorax (Fig. 3). Integument dorsally pigmented; hairs 1-P dendritic and setae 2-P with several branches regularly distributed on stem. Hairs 1-P and 2-P insert on large separated tubercles. Setae 10-P, 12-P, 9-M, and 10-M simple, 3-T palmate with simple leaflets.

Abdomen (Figs. 3 and 4). Integument hyaline or pigmented. Main tergal plate of segment VIII roughly hexagonal. Abdominal palmate setae with simple lanceolate leaflets; III-VII segments show one accessory plate on the main tergal plate; comb shows 12-15 teeth. Saddle moderately pigmented and lightly spiculate. Setae 1-X slightly longer than saddle, simple or divided into two branches.

Adult Female. Head. Vertex with frontal tuft with long white setae, anterior region with white erect scales, posterior region with black erect scales. *Clypeus* black without scales. *Labium* (length ≈ 2.1 mm) entirely black with paler labela. *Maxillary palps*

(length ≈ 1.9 mm) black from segment one to and including most of segment 4, apex of segment four and segment five entirely white. *Antenna* (length ≈ 1.6 mm) with black and bared pedicelle.

Thorax. Integument black. *Scutum* with a large median brown-gray line between the dorsocentral seta rows; sparsely covered with fine piliform brown scales. *Scutellum* without scales and with long dark setae. Dark setae on acrostichal, dorsocentral, lateral, pre-cutal, scutal, antealar and supraalar areas. Mesopostnotum and postpronotum bare. Antepronotum with dark setae laterally. Pleura with bared meskatepisternal, prespiracular and mesepimeral areas. *Wings* (Fig. 5): length ≈ 3.5 mm, costa mainly dark, with pale scales on the presector, sector, subcostal and subapical areas. Radius and R_1 vein with pale spots on basal, presector, sector, subcostal and subapical preapical areas; presector and sector pale spots of Radius vein are wider than those on the costa. R_{2+3} vein with pale spots on its basal extremity and on the fork. R_2 and R_3 veins predominantly dark scaled. R_{4+5} widely dark, with pale scales only located on its basal extremity. Median vein mainly dark scaled, pale scales occurring on basal extremity of the stem and on the fork. M_1 and M_2 veins entirely dark scaled. Stem of CuA vein shows succes-

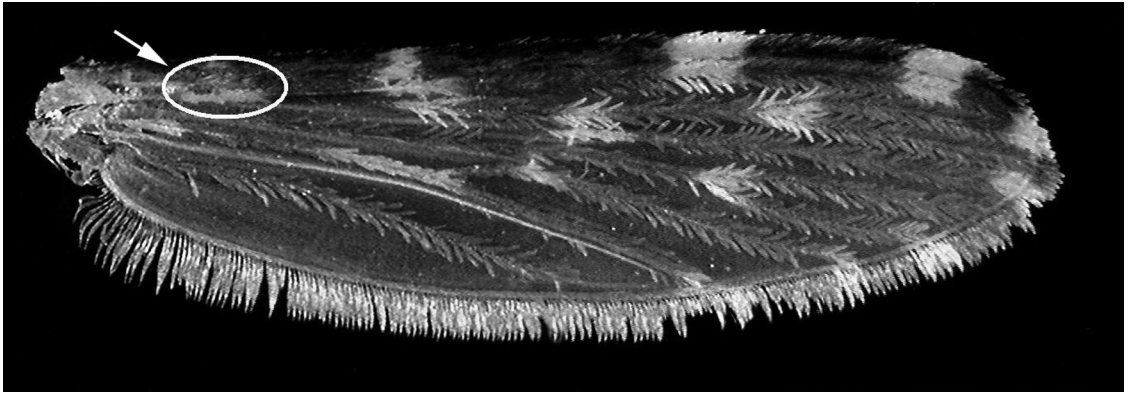


Fig. 5. Female wing of *An. ovengensis* (arrow shows white scales widely spread out on the costa and vein one presector areas).

sively a basal pale spot, a dark area and a large pale-scaled area extending up to M_{3+4} vein insertion. M_{3+4} vein with a small pale spot near the stem turn. CuP dark. Anal (IA) vein is entirely dark scaled. Alar fringe with one apical pale spot encompassing apex of R_1 and R_2 veins, and four pale spots located respectively on the apex of R_{4+5} , M_2 , M_{3+4} and CuP veins. *Halter*: pedicel and scabellum mainly dark, capitellum dark scaled. *Legs*: forecoxa, midcoxa and hindcoxa with dark setae. Femora, tibiae and tarsomeres mainly dark scaled, little clear areas are present on each segment extremity.

Abdomen. uniformly black with long dark setae, no scale overlay.

Bionomics. The biology of this new species is largely unknown. However, female *An. ovengensis* were collected on human volunteers during night catches. Human biting rate ranged from 50 to 300 bites per human per night, especially on the edges of rivers. *An. ovengensis* was rare from indoor collections, suggesting exophilic habits. Live *Plasmodium* sporozoites were directly observed in the salivary glands of 11 of 247 dissected females (4.4%) collected in Nyabessan in December 2002 (long dry season). *Plasmodium falciparum* infection rates determined by circumsporozoite protein enzyme-linked immunosorbent assay (Burkot et al. 1984) in a total of 724 mosquitoes ranged from 0.4 to 1.9% during 2002. Larval stages of *An. ovengensis* were collected along running streams and rivers, in rock and plant shelters, sometimes associated with *An. moucheti* and/or *An. obscurus*. In Afan-Essokié (≈ 50 km from Oveng), *An. ovengensis* was found once in the same larval development site with *An. carnevalei*.

Distribution. Specimens of *An. ovengensis* were collected along the running streams and rivers of southern areas in Cameroon. The species probably extends to the whole Ntem basin, and other humid equatorial areas of Cameroon, Equatorial Guinea, and Gabon.

Type Material. The type series were collected landing on volunteers near the rivers (breeding places), in the type location Oveng ($2^\circ 10' N, 10^\circ 30' E$) and in a nearby village Nyabessan ($2^\circ 10' N, 10^\circ 40' E$), ≈ 5 km from Oveng, downstream along the Ntem River.

HOLOTYPE: 1 ♀, Oveng, near Campo, 24-V-2000, Awono-Ambene.

PARATYPES: 4 ♀♀, Nyabessan, 20-VI-2001, Awono-Ambene.

Dichotomous Identification Key for Fourth Stage Larvae of *An. nili* Group. The classification of the fourth stage larvae of *An. nili* into the section III, according Gillies and De Meillon (1968) and Gillies and Coetzee (1987), is based on the following characters: distance between both 2-C setae equal or greater than distance between 2-C and 3-C setae; setae 3-C simple or with < 8 branches; setae 2-C strongly branched apically. We propose morphological characters to distinguish larvae of the *An. nili* group in Cameroon. Fourth instars of *An. carnevalei* are not described. However, we propose in this key some morphological features allowing identification of local *An. carnevalei* specimens from Afan-Essokié ($2^\circ 20' N, 10^\circ 00' E$).

1. Both long setae 9-M and 10-M simple or occasionally one split; filaments of abdominal palmar hairs (setae 1) long and drawn out:
 - YES 2
 - NO *An. rufipes* (in part)
2. Setae 1-P greatly flattened, mounted on well formed basal tubercles:
 - YES 3
 - NO *An. wilsoni* - *An. lovettae*
3. Setae 3-C very short, one quarter or less length of 2-C setae; branches of 2-C setae arising from both sides of their stem:
 - YES Anophelines other than *An. nili* group (Gillies and Coetzee 1987, pp 40–41)
 - NO 4
4. Setae 3-C as long as setae 2-C and setae 4-C extending up to or beyond bases of setae 2-C:
 - YES *An. somalicus*
 - NO 5
5. Setae 1-P dendritic:
 - YES *An. ovengensis* n.sp.
 - NO 6
6. One accessory abdominal plate present on IV–VII segments:
 - YES *An. nili* s.s.
 - NO *An. carnevalei*

Dichotomous Identification Key for Adult Female of *An. nili* Group

An. nili belongs to section XI, according to Gillies and De Meillon (1968): no scales on abdominal segments, legs entirely dark, wing with one pale spot on M₃₊₄ vein. We propose the following morphological characters to be used for proper identification of adult females within the *An. nili* group in Cameroon.

1. Maxillary palps with a pale apex, no other pale bands:
 - YES 2
 - NO Anophelines other than *An. nili* group (see Gillies and Coetzee, 1987, pp 34–35)
2. Pale fringe spots present opposite veins R₄₊₅, M₂, M₃₊₄ and CuP:
 - YES 3
 - NO *An. nili* s.s.- *An. somalicus*
3. Presector pale spots spread widely over the costa and radius vein:
 - YES 4
 - NO *An. nili* "Congo form"
4. R₂ and M₁ veins with a median white scaled area:
 - YES *An. carnevalei*
 - NO *An. ovengensis* n.sp.

Discussion

Based on morphology, bionomics (this study), and genetic evidence (Kengne et al. 2003), we propose to elevate the formerly described *An. nili* "Oveng form" to specific rank, and we name it *Anopheles ovengensis*, from its type locality. *An. ovengensis* thus is the fourth member of the *An. nili* group, together with the formerly acknowledged *An. nili* s.s. (and its clear-winged variant Congo), *An. somalicus*, and *An. carnevalei* (Gillies and De Meillon 1968, Rivola and Holstein 1957, Brunhes et al. 1999).

An. ovengensis females closely resemble *An. carnevalei* and the Congo form of *An. nili*, with white scales mainly spread out on veins and on the alar fringe of the wings. However, presector white areas on costa and radius vein are especially large in *An. ovengensis* and *An. carnevalei*, whereas they are narrow or absent in the Congo form. There are no intermediate white scales on veins R₂ and M₁ of the wing of *An. ovengensis*, as observed in *An. carnevalei*. Intraspecific variation occurred in *An. ovengensis*, some females showing no or very faint interruption between presector and sector pale spots on the costa and subcosta; the size of the pale spot on the CuA vein was also variable. However, this would not hamper correct identification, and adult *An. ovengensis* can thus readily be distinguished morphologically from all other members of the *An. nili* group with the identification key to the females we provide. However, identification at larval stages based solely on morphological characters is unreliable because *An. ovengensis* looks very similar to *An. nili* s.s. Validation of the last step of the morphological key for fourth instars we provide is still required. The recently published PCR-based diagnostic tool (Kengne et al. 2003) allowing reliable identification of all members of

the *An. nili* group at all their development stages should then be used to verify initial morphological identification. This is highly recommended and should now be required in all studies dealing with the *An. nili* group.

The observation of live *P. falciparum* sporozoites in *An. ovengensis* mosquitoes suggests its probable role in human malaria transmission, at least in villages located near rivers in southern Cameroon. Further data are needed on the biology, ecology, geographic distribution, and population dynamics of this new human malaria vector to better assess its importance in human *Plasmodium* transmission.

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