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ORIGINAL RESEARCH ARTICLE

Insecticides susceptibility of *An. melas* and its morphological discrimination with its sympatric siblings using the biometric palps technique

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Abstract

In the central western Senegal, great progress has been made against malaria following the successful implementation of effective malaria control interventions, including Indoor Residual Spraying (IRS) and Seasonal Malaria Chemoprevention (SMC). However, residual transmissions are still occurring in several hotspots involving some secondary vector species, such as *An. melas*. This study was undertaken in central and western costal area of Senegal, to provide the first data on the insecticide susceptibility of local *An. melas* population using WHO test kits, the allelic frequencies of the Kdr and Ace-1^R mutations using qPCR and also re-evaluate the palp biometry technique as a proxy to discriminate *An. melas* from other freshwater species within the *Gambiae* complex. Insecticide susceptibility test revealed a susceptibility of *An. melas* to Pirimiphos-methyl, and Bendiocarb, the two main non-pyrethroid insecticides recommended by the WHOPES for use in public health. The molecular characterization of the *Kdr* and *Ace*-1 target site mutations revealed the absence of both mutations. The biometric palps technique has been a valid method for species diagnose between *An. melas* and its freshwater sibling. Indeed, while the former species collected exclusively from salty breeding sites (with a level of salinity above 21 g/l), consistently displayed a palpal index ≥0.81; the latter, sampled for breeding site of low salinity level (up to 3.6 g/l) and subsequently mainly identified as *An. arabiensis* and in a lesser extend as *An. gambiae*, presented a palpal index less than 0.81. This study has re-evaluated and validated the palps biometric technique as a morphological tool for the identification of *An. melas*, which population still susceptible to main insecticide used in public health and revealed the absence of KDR and Ace-1 mutations. The data provided here can help the Senegalese NMCP to better target and efficiently control *An. melas* populations in malaria hotspot, where they contribute to maintain residual transmission hampering the malaria elimination goal.

Keywords *An. melas* · Palpal index · Insecticides susceptibilities · Senegal

Background

Senegal is one of the West African malaria-endemic countries with the potential to eliminate malaria in few eligible areas following several control efforts. Indeed, the country has globally recorded a positive trend regarding major malaria indicators, with an important decrease in the malaria-related proportional mortality of about 12% (PNLP. 2018). Despite an unprecedented opportunity to eliminate malaria where possible, there is an urgent need to gather updated data on different vectors involved in transmission, their biology and their ecology to better target vector control interventions and use in a cost-effective way the limited resources available. In western central Senegal, great progress has been made against malaria with the successful implementation and scale-up of several effective control interventions, including Indoor Residual Spraying (IRS) and Seasonal Malaria Chemo prevention (SMC). However, several residual transmission areas (also so-called hotspots) are still being recorded, and preliminary studies have revealed that in the area of interest, *An.*

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arabiensis, An. coluzzii and *An. melas* are the main vectors species maintaining the transmission (Sy et al. 2018). While the former two species have been widely studied revealing their widespread distribution across the country as well their predominant role as main malaria vector, *An. melas*, previously less studied in term of vector bionomic and insecticide susceptibility status than its sibling, has now started to attract more the spotlight only very recently (Sy et al. 2018). *An. melas*, is one of the halophilic species of the Gambiae complex distributed along the coastal brackish areas of the Western and Central Africa (Diagne et al. 1994; Diop et al. 2002). By definition the sibling species are closely related species that are morphologically indistinguishable using classic taxonomic methods (Coluzzi et al. 1985). Therefore, cytogenetics and molecular methods have been developed and widely used to discriminate species within vector complexes and groups (Scott et al. 1993). Despite the biometry method based on palps band pattern length has shown a potential to differentiate *An. gambiae* s.l. freshwater and the brackish water species, it has been scarcely or not used at all. The method is based on the calculation of the ratio between the length of the 4th and 5th maxillary palps segments comparatively to the 3rd one (Coluzzi 1964). In a context where the possibility for community-based surveillance approach is investigated and where the lack of electricity could be a big challenge for routine monitoring. Therefore using the palpus biometric technique approach could be an interesting proxy to quickly identify *An. melas* in residual transmission areas where it could play a major role.

Moreover, due to its presumed secondary role in malaria transmission, *An. melas* has been less studied regarding its susceptibility to insecticide than *An. gambiae* and *An. arabiensis*. To our knowledge, only few studies have investigated the susceptibility of *An. melas* populations to insecticide used in public health (Akogbéto 1995; Gnanguenon 2015).

During the last decades, there is a renewed interest for malaria elimination where feasible. However, the progress toward the elimination may be hampered by the residual transmission maintained in few areas by previous secondary/focal vector species, including *An. melas*. In this context, it's crucial to generate updated data on the bionomics, the insecticide resistance/susceptibility of such a species, particularly in areas of low transmission, to better understand their biology and role in the residual malaria transmission as recorded in the coastal environment as well as their status of insecticide susceptibility to better guide current and future vector control interventions.

This study was conducted in the coastal zone of western-central Senegal to provide updated data on the susceptibility of *Anopheles melas* populations to main public health insecticide and the allelic frequencies of the *Kdr* and *Ace* 1^R mutations among their local populations. But also, to verify the reliability of the palpus biometry approach to discriminate sympatric *Anopheles melas* specimens from its freshwater sibling.

Methods

Study area

The study was conducted in three villages of the central-western Senegal, Keur Martin (14°24′29"N; 16°34′29"W), Diakhanor (13°58′41"N; 16°45′45"W) and Mbind Coly (14°17′10"N; 16°54′30"W), located respectively in the health districts of Diofior, Mbour and Fatick (Fig. 1). The first study village (Keur Martin) was chosen in a continental area, while the two latter (Diakhanor and Mbind Coly) are located in the coastal zone. All the three sites are surrounded by large expanses of brackish water influenced by tidal movements (Fig. 1).

The study was carried out toward the end of the rainy season (October–November) when the populations of *An. melas* are expected to be the highest since no freshwater body persists and in those remaining the salinity increase as the water evaporate making them most suitable for *An. melas* larvae.

The study area belongs to the Sudan Bioclimatic Region, characterized by a long dry season lasting from November to June and a short rainy season from July to October. The average annual rainfall recorded over the last 15 years varied between 400 and 600 mm, with an average temperature of 28 °C (Sy et al. 2016).

Characterization of breeding sites and sampling of larval population

All the surface water bodies encountered inside and around the three study villages were prospected, then characterized when found harboring anopheline larvae. The conductivity and salinity of water were measured for each positive breeding site using a Water Monitoring Kit. Since the study mainly aimed to distinguish the salty species of the complex, namely *An.* melas, from its freshwater sibling, we mainly focused on the level of salinity of breeding sites which were split into low salinity breeding sites (≤3.6 g/l) and high salinity breeding sites (≥21 g/l). The larvae found in both type of breeding sites were sampled using the dipping method, sorted then reared separately.

Measure of the palpal index for the morphological discrimination of species

All the specimens of *An. gambiae* s.l., were collected from two type of breeding sites: breeding with the low salinity (≤3.6 g/l) were classified as freshwater breeding sites and

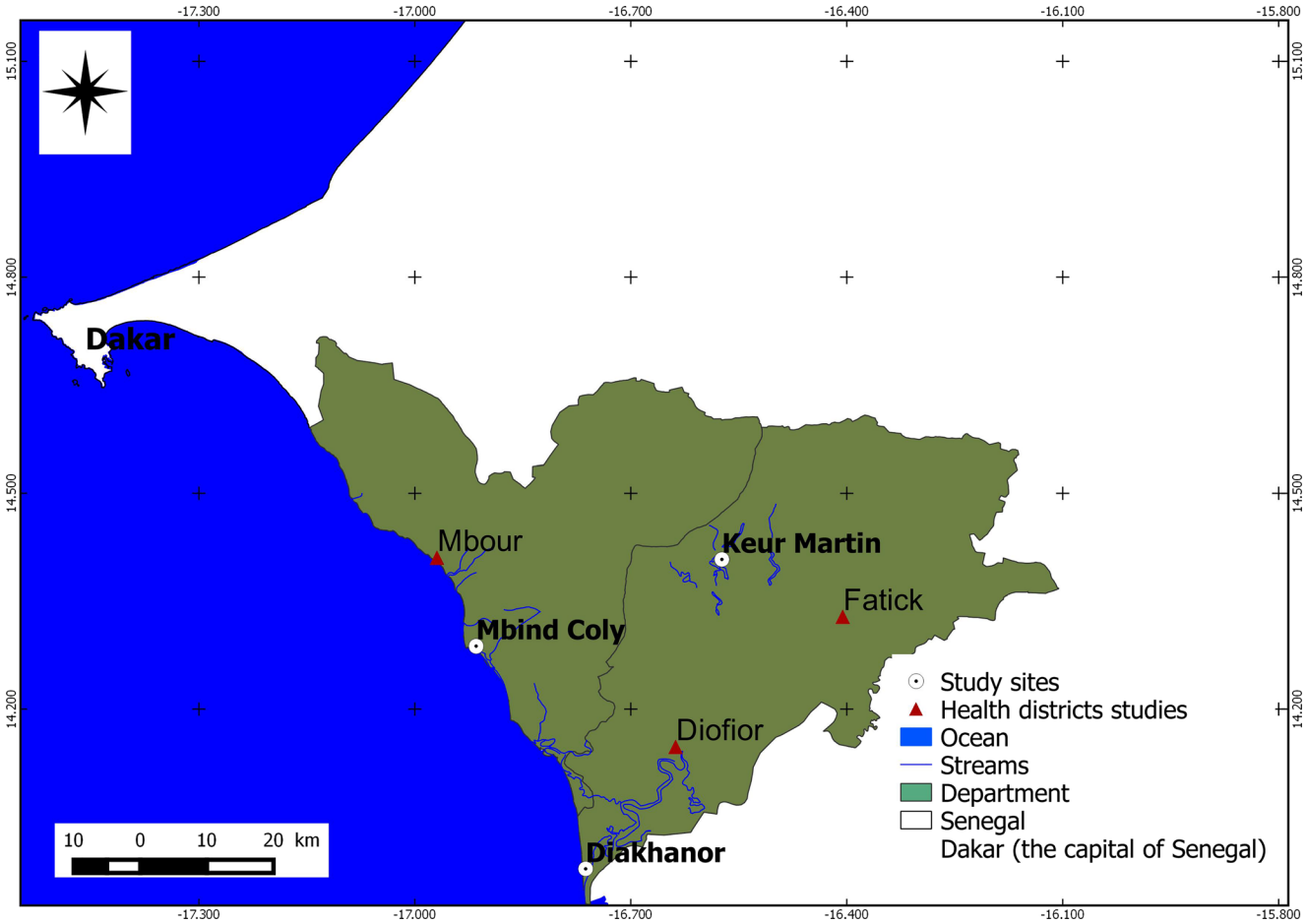

Fig. 1 Study area (central-western Senegal/west Africa)

those with high salinity (≥21 g/l) were considered as salty breeding sites. Upon collection mosquitoes were firstly identified using a morphological dichotomic key (Gillies and Meillon (1968)), then sorted according to the breeding sites from which they were collected from (freshwater breeding sites vs salty breeding sites). Then the palpal index of specimens randomly selected from both groups was determined as described by Coluzzi (1964) to discriminate the *An. gambiae* s.l. freshwater species from their brackish water siblings. The palpal index represents the quotient between the length of segments IV and V (IV + V) by the length of segment III of maxillary palps (Faye 1987; Faye et al. 1994). Prior the determination of palps index, heads of sampled females were cut off then immersed into individual tubes containing lactophenol for lightening of the maxillary palpus. After 48 h, heads were individually mounted onto a slide then fix with a drop of Polyvinyl Alcohol (PVA). The palps were cautiously separated from the proboscis while mounting heads, then measured using a micrometer associated with the microscope's objectives (Fig. 2). Both palps of each head were measured to calculate the average length of segments of interest to determine the palpal index of each specimen (Faye 1987). Specimens with an index ≥0.81 were classified as brackish water species (*An. melas*) and those with an index <0.81 among freshwater species (potentially *An. gambiae, An. coluzzii* and *An. arabiensis*) as in Faye et al. (1994), Faye et al. 1994).

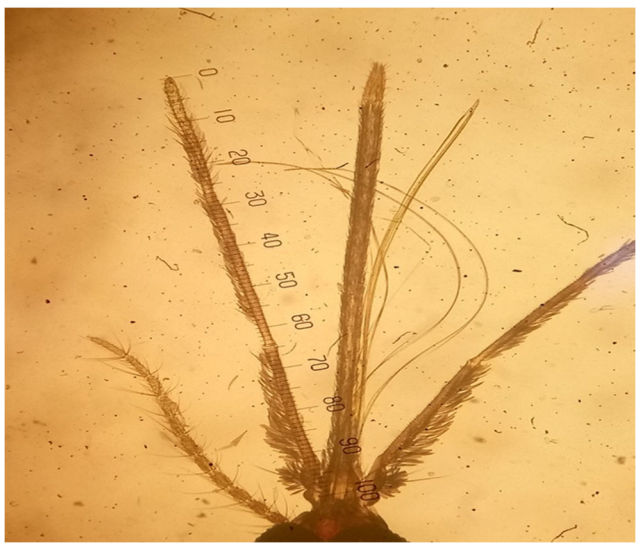

Fig. 2 Measurement of the palpal index (alignment between micrometer and mosquito's palp using a microscope)

Insecticide susceptibility tests

The insecticide susceptibility tests were carried out using the WHO test kits for adult mosquitoes (OMS 2017b). The study mainly targets *An. melas* populations, therefore the tests were performed only on adult females collected from the high salinity breeding sites (≥ 21.4 g/l), only found in Mbine coly in the coastal area. Unfed, 3 to 5 days adult females were exposed to Bendiocarb 0.1% and Pirimiphos-methyl 0.25%, which are previously and currently used in vector control intervention in the study area, respectively (Sy et al. 2019). During the test, optimal rearing conditions of a relative humidity of 75 ± 5% and a temperature of 28 ± 2 °C were maintained and monitored using a thermo-hygrometer. For each molecule, four batches of 20–25 non-blood fed females were exposed to insecticides and two batches of 20–25 non-blood fed mosquitoes were used as a control. During the test, the number of mosquitoes knocked down was monitored and recorded after 10, 15, 20, 30, 40, 50, and 60 min post-exposure, then transferred to holding tubes and provided with cotton pads soaked with 10% sugar solution. The differed mortality rates among both test and control groups were recorded 24 h post-exposure. Tests were validated on the control group and results were interpreted according to the WHO criteria (OMS. 2017a). The test was validated without correction, if the mortality in the control is below 5%. But discarded when the control mortality is above 20%. For control mortality between 5 and 20%, the mortality observed in the test group is corrected using the Abbott's formula (OMS. 2017a). Based on the WHO criteria, the tested population was recorded as resistance when the mortality rates in the test batch is below 90%. While mortality rates above 98%, was indicative of susceptible population. Finally, mortality rates between 90 and 98% suggest the possibility of resistance that needs to be verified (OMS. 2017a). For each molecule, a sub-sample of at least 30 specimens of *An. gambiae* s.l. specimens, randomly selected, was subsequently identified morphologically for the confirmation of the taxon, then by PCR for the *An. gambiae* s.l. species diagnostic as detailed below.

Molecular identification of the *An. gambiae* complex species and characterization of resistance mutations using TaqMan assays

The genomic DNA was extracted from legs and/or wings of individual specimen, selected from samples used for the palpal index determination and from the susceptibility tests, using the Chelex method as described by Musapa et al. 2013. The molecular identification of the members of the *An. gambiae* complex was performed by the PCR from Wilkins et al. (2006).

The putative insecticide resistance mutations, including the Ace-1-119S and the knockdown resistance (kdr) mutations (Vgsc-1014F or kdr-west and Vgsc-1014S or kdr-east) were characterized using the Taqman polymerase chain reactions (qPCR). For the Kdr mutations, two Taqman qPCRs were performed separately using the same set of Forward (5'-CATTTTTCTTGGCCACTGTAGTGAT-3′), and Reverse (5'-CGATCTTGGTCCATGTTAATTTGCA-3′) primers and the same wild-type probe (5'-CTTACGACTAAATTTC-3′) labelled with VIC dye, but two different sequences respectively for the Vgsc-1014F mutation or west-kdr (5'-ACGACAAA ATTTC-3′) and Vgsc-1014S mutation or east-kdr (5'-ACGACTGAATTTC-3′) both labelled with 6-FAM dye. Each PCR reaction tube contain 10 µl: 5 µl of SensiMix; 0.125 µl of Primer/probe; 3.875 µl of sterile water and 1 µl of genomic DNA. The cycling conditions was 10 min at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s and the fluorescence measured at the end of each cycle) (Bass et al. 2007). The Ace-1R assay to detect the G119S mutation gene encoding the acetylcholinesterase enzyme (ace) required two standard primers ace1-Forward (5'-GGCCGTCATGCTGT GGAT-3′) and ace1-Reverse (5'-GCGGTGCCGGAGTA GA-3′) and two TaqMan probes VIC-5'-TTCGGCGG CGGCT-3′ respectively for wild-type allele and 6FAM-5'-TTCGGCGGCAGCT-3′ for the resistant allele. This reaction uses the same concentrations and thermal conditions as for the kdr assay (Bass et al. 2010).

Results

Distribution of the breeding

The survey of the larval breeding sites revealed that only three anopheline breeding sites were found in the coastal area with the low salinity breeding site located in the locality of Diakhanor, while those highly salty were encountered only in Mbine coly. None positive breeding site has been found in Keur Martin in the continental area. Therefore, subsequent analyses were done only with larval populations collected from the coastal zone.

Morphological identification using the palpal index approach

A total of 181 specimens (94 from freshwater breeding site and 87 from the salty one) were used for the morphological identification using the palpal index approach. The measurements of the palpal indexes of specimens collected from the freshwater breeding sites showed an overall mean palpal index of 0.76, ranging from 0.68 to 0.88, with 92.6% (87/94) of the freshwater specimens displaying a palpal index inferior to the threshold (0.81) (Table 1). Conversely, the palpal indexes of specimens collected from salty breeding sites and lately identified as *An. melas*, displayed a mean papal index of 0.86,

Table 1 Ratios between the combined lengths of the 4th and 5th segments and the 3rd segment of the maxillary palps in adult females from low (≤3.6 g/l) vs high (≥21 g/l) salinity breeding sites

Salinity range (g/l)	Total studied	Index < 0.81	Index ≥ 0.81
≤3.6 g/l	94	87 (92.6%)	7 (7.4%)
≥21 g/l	87	4 (4,6%)	83 (95,4%)

ranging from 0.70 to 0.97, with 95.4% (83/87) of salt-tolerant individuals displaying an index ≥0.81 (Table 1).

Insecticide susceptibility of *An. melas*

Overall, 112 and 127 adult mosquitoes from high salinity breeding sites (≥ 21.4 g/l) were exposed to Pirimiphos-methyl 0.25% and Bendiocarb 0.1%, respectively. The results revealed that *An. melas* was susceptible to both molecules with 100% and 99.2% mortality, respectively for the Pirimiphos-methyl 0.25% and the Bendiocarb 0.1% (Table 2).

Molecular identification of specimens from low salinity larval breeding sites

Overall 173 specimens of the *An. gambiae* s.l. were identified by PCR. Of these 88 were collected from the freshwater breeding sites and the 85 remaining for the salty breeding sites.

The molecular identification revealed that the *An. gambiae* s.l. populations from freshwater breeding sites were almost all exclusively represented by *An. arabiensis* (97.7%), found together with *An. gambiae* at low proportion (2.3%) (Table 3). While the salty breeding sites, only found in the coastal village of Mbine coly, harbored exclusively *An. melas,* (Table 3).

Prevalence of KDR et Ace-1 mutations among *An. melas* population

The molecular characterization of the Kdr and Ace-1 target site mutations of all *An. melas* specimens exposed to pirimiphos-methyl and Bendiocarb revealed the absence of both mutations in all study population, where only the wild types kdr (L1014L) and Ace-1 (G119G) alleles were found (Table 4).

Discussion

Molecular identifications of the *An. gambiae* s.l. species, revealed the exclusive presence of *An. melas* larvae in the salty breeding sites of salinity level above 21 g / l. While in those much less salty (up to 3.6 g/l) were colonized mainly by *An. arabiensis* (97.7%), and scarcely by *An. gambiae* (2.3%). These results are consistent with the known larval ecological preferences of the above species. The exclusive presence of *An. melas* in salty breeding sites is consistent with its halophilic status as reported elsewhere (Attolou et al. 2016). Indeed, *An. melas* is one of the salt-tolerant species of the Gambiae complex distributed in the coastal areas of the Western and Central Africa. Its larvae grow usually in salty water with salt concentration close to the seawater salinity (Coluzzi 1965). During the study, *An. melas* has been found in sympatry with *An. arabiensis* and *An. gambiae* s.s. with similar proportions as previously reported in the mangrove forest of the Saloum Delta (Diop et al. 2002). The two later species are widely distributed in the Afro-tropical region where they are often found in sympatry with their other sibling of the Gambiae complex (Chauvet et al. 1969). In addition, both species display an ecological polymorphism across their distribution range regarding their salinity tolerance. Indeed, Robert et al. (1998), using a co-inertia analysis between the numbers of mosquitoes and the physicochemical variables of the water, have shown that the presence of *An. arabiensis* larvae in prospected breeding sites was associated with high values for carbonates, pH, and salinity. More recently, Tene Fossog et al. (2015), have modeled using a large set of occurrence records and eco-geographic information that the salinity tolerance of both *An. coluzzii* and *An. gambiae* s.s. presumably reflect their adaptive response to osmotic stress from anthropogenic pollutants, especially in urban localities of the the Western and Central Africa. *An. melas* has been already reported in sympatry its freshwater siblings across different areas of Senegal (Sy et al. 2018; Pages et al. 2008; Diop et al. 2002; Faye et al. 1994; Faye 1987) and across the western Africa (Attolou et al. 2016; Diabate et al. 2002; Odetoyinbo 1969). In the mangrove area of the Saloum Delta in Senegal Diop et al. (2002) have previously reported the sympatric occurrence of *An. melas* with *An. arabiensis*, with *An. melas* playing a secondary role in malaria transmission. Indeed, due to its pronounced zoophilic behavior and

Table 2 Mortality rate of *An. melas* from high salinity breeding sites, after 24 h exposure to Organophosphates (Pirimiphos-methyl 0.25%) and Carbamates (Bendiocarb 0.1%)

Salinity range	Insecticides tested	Total tested	Dead	Mortality (%)
≥21 g/l	Pirimiphos-methyl 0.25%	112	112	100
	Bendiocarb 0.1%	127	126	99.2

Table 3 Species composition by salinity range of breeding sites

Salinity range *(g/l)*	*An. arabiensis*	*An. coluzzii*	*An. gambiae*	*An. melas*	Total
≤3.6 g/l	86	0	2	0	88
≥21 g/l	0	0	0	85	85

more especially to its short lifespan, *An. melas* has been usually described as a very poor malaria vector (Lemasson et al. 1997; Faye et al. 1994; Faye 1987). However, even if lesser anthropophilic than its other siblings such as *An. gambiae* and *An. coluzzii, An. melas* easily enters homes to feed on human in the absence of animals (Reddy et al. 2011). This behavior has been already reported from the neighboring country of The Gambia, where up to 80% of *An. melas* samples collected along the Gambia river fed on human (Caputo et al. 2008).

Due to the lack of sufficient number of sample due the successive implementation of successive IRS campaigns using non-pyrethroids insecticide, such as the Pirimiphos-methyl and the Bendiocarb, the study populations collected from salty breeding sites (≥ 21.4 g/l) across the study area, subsequently confirmed as exclusively *An. melas*, were exposed only to the two above-mentioned molecules. The bioassays results revealed that *An. melas* populations were susceptible to both tested insecticides. Despite preliminary, the data presented here provide the first and updated information on the status of *An. melas* populations from Senegal to the two main non-pyrethroid insecticides recommended by the WHOPES for use in public health. Furthermore, to our knowledge, no extensive or only few previous data exist on *An. melas* status to insecticide across its range of distribution throughout the Western African coastal environment. Our results are consistent with the few previous existing data which have reported the full susceptibility of *An. melas* populations to tested insecticides (Bendiocarb and pyrethroids) in Benin (Gnanguenon 2015; Akogbéto and Yakoubou 1999).

None of the main target site mutations responsible for either Organophosphate/Carbamate (119S) or DDT-pyrethroids (1014F and 1014S) cross-resistances were seen among the studied *An. melas* populations in the central western of Senegal. Some studies carried out in Senegal on *An. arabiensis* reported full resistance to pyrethroids and DDT with a predominance of KDR East mutation (Dia et al. 2018; Niang et al. 2016). These results may be explained by the recent implementation of a community-based Indoor Residual Spraying (IRS) intervention with pirimiphos-methyl in the study area to manage the widespread of pyrethroid resistance among targeted vector populations (Sy et al. 2019).

The morphological taxonomy using the palpal index has been proposed earlier as a valid approach, however scarcely and no more used, to discriminate between the freshwater and brackish water species of the *An. gambiae* complex (Coluzzi 1964). The method has been successfully used in the present study to discriminate *An. melas* (colonizing salty breeding site) from its freshwater siblings (*An. gambiae, An. coluzzii* and *An. arabiensis*). However, less than 8% of freshwater as well as salty breeding sites populations displayed respective palpal indexes of ≥0.81 and < 0.81, stressing the need for caution in the use of the method and a PCR confirmation whenever possible. Moreover, the palpal index of the brackish water populations ranged from 0.70 to 0.97, with the upper limit and the average index (0.86) slightly higher than previously defined by Coluzzi (1964). However, 96.5% (84/87) of individuals have an index between 0.78 and 0.95, corresponding to the interval defined by Coluzzi (1964). This range is also close to that reported by Faye in 1987 in the upstream area of the Bignona anti-salt dam (Faye 1987). Overall, the results of the study clearly show that brackish water species have a palp score greater than 0.81 while freshwater species are below the 0.81 threshold and this approach could be an interesting proxy to quickly identify *An. melas* in areas where there is a lack of electricity limiting the use of current molecular methods.

Molecular analysis revealed the exclusive presence of *An. melas* in salty larval breeding sites and its absence freshwater breeding sites. Moreover, molecular identification who is the most optimal method to separate *An. melas* from the other members of the complex (Palsson and Pinto 1998), revealed that freshwater population with a palpal index less than 0.81 consisted mainly of *An. arabiensis* and in a lesser extend of *An. gambiae*.

Table 4 Detection of KDR and Ace-1 mutations among *An. melas* species tested with Pirimiphos-methyl and Bendiocarb

		KDR genotype (%)						Ace-1 genotype (%)		
Molecule tested	Effectif	L1014L	L1014F	L1014S	F1014S	F1014F	S1014S	G119G	G119S	S119S
Pirimiphos-methyl 0.25%	55	100%	0%	0%	0%	0%	0%	100%	0%	0%
Bendiocarb 0.1%	30	100%	0%	0%	0%	0%	0%	100%	0%	0%

Conclusion

The present study has re-evaluated and validated the accuracy of the biometric palps technique for a morphological identification of *An. melas* as defined by Coluzzi (1964). The study provided also the first data on the susceptibility of *An. melas* to mains non-pyrethroid insecticides families used in public health in Senegal. The data reported here were made available to the Senegalese NMCP to guide targeted and evidence-based control intervention against this species, suspected as one of the vector species maintaining local residual malaria transmission in the hotspots across the study area.

Authors' contributions OS, EAN, LK, OG and OF designed the study. OF, OS and OG supervised the study. OS carried out the field collections and performed the experiments with AN, MAN and PCS. OS, EAN, MN, AKD, OKG and OF contributed toward data analysis. OS, EAN, MN, AKD, BS and MAN analysed the data and wrote the manuscript. All authors read, and approved the final manuscript.

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Compliance with ethical standards

Ethics approval and consent to participate This study was approved by the Ethics Committee of University Cheikh Anta Diop of Dakar, Senegal.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

Abbreviations IRS, Indoor Residual Spraying; SMC, Seasonal Malaria Chemoprevention; WHO, World health organization; Kdr, Knock-down resistance; Ace-1, Acetylcholinesterase (Ace-1) target site mutation; WHOPES, World Health Organization Pesticide Evaluation Scheme; NMCP, National Malaria Control program; PCR, Polymerase chain reaction; PVA, Polyvinyl Alcohol; DNA, desoxyribonucleic Acid; DDT, Dichlorodiphénytrichloroéthane

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