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Écologie et taxonomie intégrative des moucherons piqueurs du genre *Culicoides* Latreille (Diptera : Ceratopogonidae) en région Afrotropicale

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Femelle de *Culicoides* (Diptera : Ceratopogonidae)

"Nothing in biology makes sense, except in the light of evolution"

Theosodius Dobzhansky (1973)

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Introduction générale

La notion d'espèce est fondamentale en biologie. Les espèces sont la première mesure de la biodiversité et l'unité de référence de nombreux concepts à travers le monde (Connell, 1978; Myers *et al.*, 2000). La taxonomie primaire ou alpha-taxonomie (du grec ταξινομία *taxis*, rangement, et *nomos*, loi) est une science qui a pour objet de décrire les organismes vivants (ou ayant vécu) et de les regrouper en entités appelés taxons (familles, genres, espèces) afin de pouvoir les identifier, les nommer et enfin les classer. Ce classement implique de connaître les relations historiques qui existent entre les taxons, ce que s'attache à faire la classification systématique. Le terme taxonomie (ou taxinomie) fut inventé, par Augustin Pyrame de Candolle (1778-1841) pour définir la *théorie des classifications*, mais la classification des êtres vivants avait déjà été initiée dès l'antiquité, entre autre par Aristote (4ème siècle avant JC), puis développée par Carolus Linnaeus, appelé par la suite Carl Von Linné (1707-1778). Cette discipline est en relation avec l'évolution des critères permettant la classification d'où la notion d'homologie. Ce terme, développé par Étienne Geoffroy Saint-Hilaire (1772-1844), désigne le lien évolutif entre deux caractères (longtemps anatomiques et depuis les 30 dernières années surtout moléculaires) observés chez deux espèces différentes lorsque ce caractère est hérité d'un ancêtre commun. On parle alors de caractères homologues. Ce concept a pris une importance considérable avec l'avènement du Darwinisme. Les théories de l'évolution prédisent qu'un groupe d'organismes similaires descendent d'un ancêtre commun d'où les fondements de la phylogénie introduite par Willi Hennig (1913-1976). La systématique phylogénétique (cladistique) est une méthode de classification taxonomique des organismes basée sur leur histoire évolutive (Hennig, 1950). La cladistique hiérarchise les caractères comparés. Les êtres vivants seront regroupés dans un même groupe (appelé taxon) uniquement lorsqu'ils partagent des caractères homologues (recherche d'une ascendance commune). La première représentation détaillée de l'évolution est le fait de Ernst Haeckel (1834-1919) qui le premier publia un arbre d'évolution en 1866 dans son ouvrage '*Morphologie générale*' (Haeckel, 1866).

Pendant longtemps, la taxonomie des espèces se faisait sur des critères purement morphologiques. Etant très souvent influencés par l'environnement, les caractères morphologiques ne peuvent pas toujours à eux seuls constituer des critères suffisamment fiables pour établir des classifications reflétant les réelles relations historiques entre les taxons. Le développement et la généralisation des techniques de biologie moléculaire depuis

ces 30 dernières années ont permis d'utiliser les caractères moléculaires entrainant des révisions taxonomiques importantes. La phylogénie moléculaire s'appuie sur le principe selon lequel les séquences d'ADN des organismes vivants évoluent sous l'influence de mutations successives qui s'accumulent au cours du temps et font l'objet de processus de sélection naturelle. La parenté entre les organismes vivants est reflétée par le niveau de similarité des séquences primaires de leur ADN et de leurs protéines. Des espèces très proches ont un ancêtre commun récent, et donc peu de mutations ont eu le temps de se produire depuis qu'elles ont divergé. La phylogénie moléculaire a ainsi permis de redonner une nouvelle vision à la science taxonomique en permettant de mieux comprendre l'évolution de certains traits morphologiques, moléculaires, écologiques, comportementaux des organismes et une meilleure compréhension des processus évolutifs sous-jacents.

Aujourd'hui, dans un contexte de mondialisation des échanges de biens et des personnes et de changement climatique, la taxonomie des espèces d'arthropodes d'intérêt médical ou vétérinaire est devenu un enjeu majeur pour la surveillance de la distribution actuelle, la prévention vis-à-vis des introductions, et le contrôle des espèces d'arthropodes impliquées dans la transmission de pathogènes. En assignant un spécimen à un nom d'espèce, la taxonomie permet d'avoir accès au corpus de connaissances scientifiques comme la bio-écologie et les cycles épidémiologiques. Ces informations sont essentielles pour développer des modèles prédictifs permettant d'anticiper les crises sanitaires, et de mieux surveiller et contrôler la transmission du pathogène. Comme le disait le grand naturaliste Jean-Henri Fabre (1823-1915) dans son ouvrage, Les Souvenirs Entomologiques Livre 1, (Fabre, 1879) : « *Vainement on me dira que telle espèce a tant d'articles aux antennes, tant de nervures aux ailes, tant de poils en une région du ventre ou du thorax ; je ne connaîtrai réellement la bête que lorsque je saurai sa manière de vivre, ses instincts, ses mœurs* ».

La famille des Ceratopogonidae (Ordre : Diptera) est représentée dans le monde entier par environ 110 genres et 6 000 espèces décrites (Borkent, 2016). Les moucherons piqueurs du genre *Culicoides* Latreille, 1809 sont des petits diptères (de 1 à 4 mm de longueur) appartenant à la famille des Ceratopogonidae (Figure 1). Ce genre est présent sur toute la surface de la planète à l'exception de l'Antarctique et de la Nouvelle-Zélande (Meiswinkel *et al.*, 2004a). Le genre *Culicoides* compte environ 1 400 espèces (Borkent, 2016). Certaines espèces du genre *Culicoides* sont impliquées dans la transmission de pathogènes d'intérêt médical et vétérinaire (virus et nématodes) partout dans le monde (Mellor *et al.*, 2000; Mullen, 2009; Purse *et al.*, 2015).

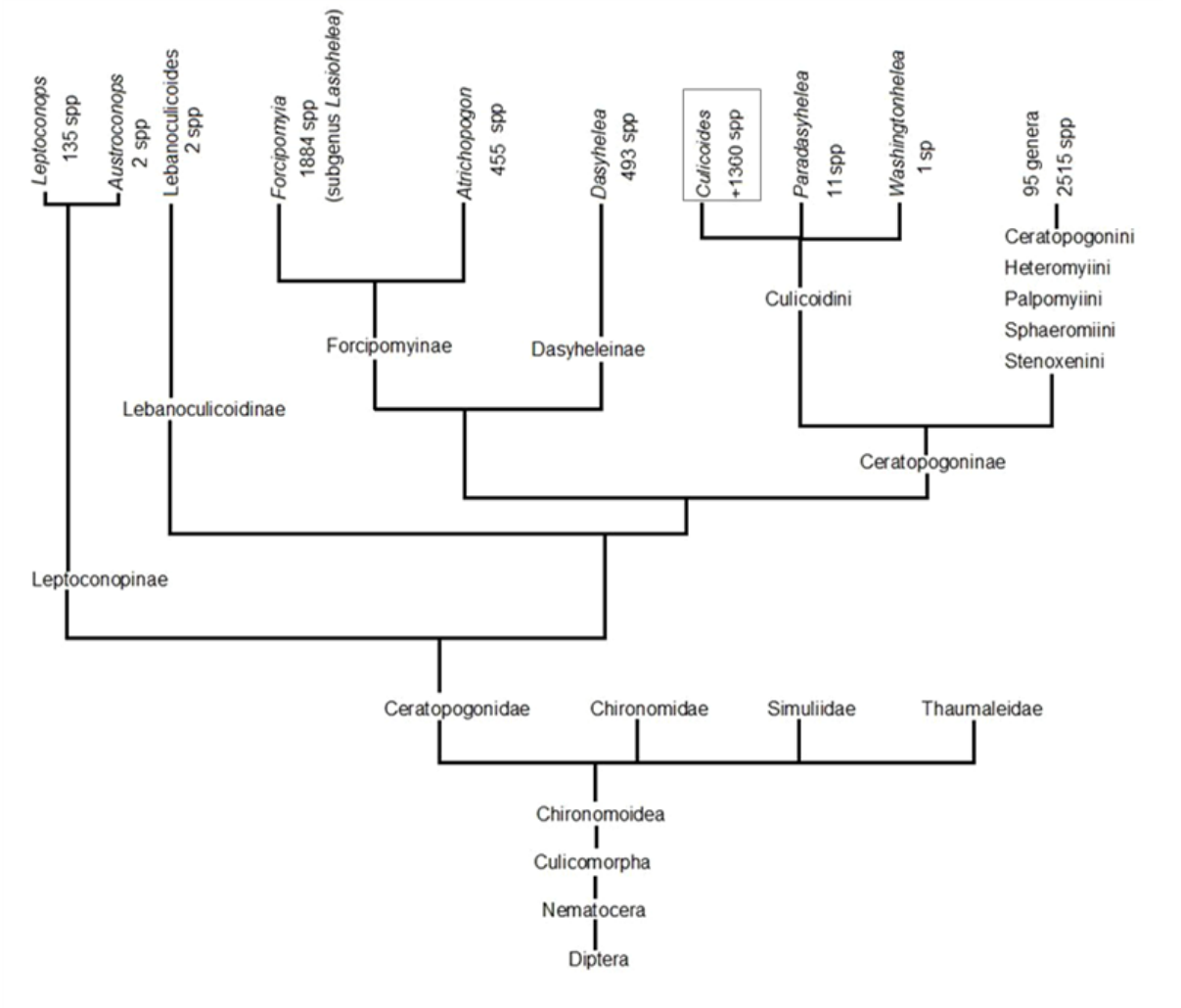

Figure 1 : Position systématique du genre *Culicoides* Latreille au sein des Diptères (Labuschagne, 2016)

En région Afrotropicale, quatre virus d'intérêt vétérinaire sont transmis par certaines espèces de *Culicoides* : les virus d'Akabane, de la fièvre catarrhale ovine, de la fièvre hémorragique épizootique transmis aux ruminants sauvages et domestiques, et de la peste équine, transmis aux équidés. La peste équine est létale pour les chevaux. Les épizooties de cette dernière sont responsables de plusieurs crises sanitaires avec des conséquences économiques importantes (Carpenter *et al.*, 2017; Purse *et al.*, 2015). Au Sénégal, l'épizootie de peste équine de 2007 a provoqué la mort de 1 169 chevaux, coûté environ 1,37 millions d'euros (Akakpo *et al.*, 2011) et affecté 26 des 34 départements de ce pays (Diouf *et al.*, 2012). Si d'importants travaux taxonomiques ont été réalisés en région Paléarctique pour décrire la faune culicoidienne (Mathieu *et al.*, 2012), celle de la région Afrotropicale reste peu étudiée avec des inventaires ou des descriptions anciennes (Cornet et Brunhes, 1994; Glick, 1990; Rawlings *et al.*, 2003).

Récemment, l'expertise de Rudy Meiswinkel et Karien Labuschagne, chercheurs à l'*Onderstepoort Veterinary Institute* (OVI) en Afrique du Sud a permis de mettre à jour la faune culicoidienne d'Afrique australe avec environ 150 espèce de *Culicoides* (Labuschagne, 2016; Meiswinkel, 1996). Suite à l'épizootie de peste équine de 2007 et dans le cadre du projet EDENext financé par l'Union Européenne et coordonné par le Cirad (EU FP7-HEALTH-2010-single-stage grant 261504 EDENext), l'Institut Sénégalais de Recherches Agricoles (ISRA) et le Cirad ont mis à jour la diversité du genre *Culicoides* au Sénégal avec 19 nouvelles espèces répertoriées portant le total d'espèces présentes au Sénégal à 53 espèces (Bakhoum *et al.*, 2013; Diarra *et al.*, 2014; Fall *et al.*, 2015a). Dans la continuité de ces travaux de recherche, le premier chapitre de revue des connaissances taxonomiques et bio-écologiques abordera plus en détail le genre *Culicoides* en région Afrotropicale au travers de quatre parties : (i) la diversité du genre *Culicoides*, (ii) l'imbroglio taxonomique et systématique de ce genre et de manière concomitante le manque d'outils d'identification morphologique, (iii) l'état des connaissances sur la bio-écologie des espèces de *Culicoides* en région Afrotropicale, et (iv) leur importance en santé publique (**Chapitre 1**).

Chapitre 1 : Le genre *Culicoides* Latreille en région Afrotropicale

La première description d'une espèce de *Culicoides* a été faite par le révérend William Derham en 1713 en Angleterre (Mellor *et al.*, 2000; Reye et Lee, 1963), la description du genre plus tard par Latreille en 1809 avec comme espèce type du genre, *C. punctatus* (Meigen) (Borkent, 2016; Mathieu, 2011). La description des deux premières espèces de *Culicoides* en région Afrotropicale, *C. herero* Enderlein et *C. schultzei* Enderlein, intervient plus d'un siècle après la description de Derham (Enderlein, 1908).

1. Diagnose morphologique du genre *Culicoides*

Les moucherons piqueurs du genre *Culicoides* sont de taille et de couleur variables allant de 1 à 4 mm, et de brun-jaunâtre à noir. Par comparaison aux autres genres de la famille des Ceratopogonidae, le genre *Culicoides* se distinguent par les caractères suivants :

- Tête (Figure 2 A) : (i) quinze articles antennaires chez la femelle (scape + pédicelle + 8 articles courts + 5 articles longs) ; (ii) le 15ème article est dépourvu de mucron (Figure 2 B) ; (iii) présence de soies sensorielles ; (iv) palpe composé de 5 articles dont les deux premiers sont soudés et le troisième présente une fossette sensorielle ;

- Ailes (Figure 2 C) : (v) présence de deux cellules radiales subégales (sauf dans le sous-genre *Trithechoides*) ; (vi) nervure transverse (r-m) est toujours présente ;
- Pattes (Figure 2 D) : (vii) l'empodium porté par le dernier segment du tarse de la patte postérieure est rudimentaire.

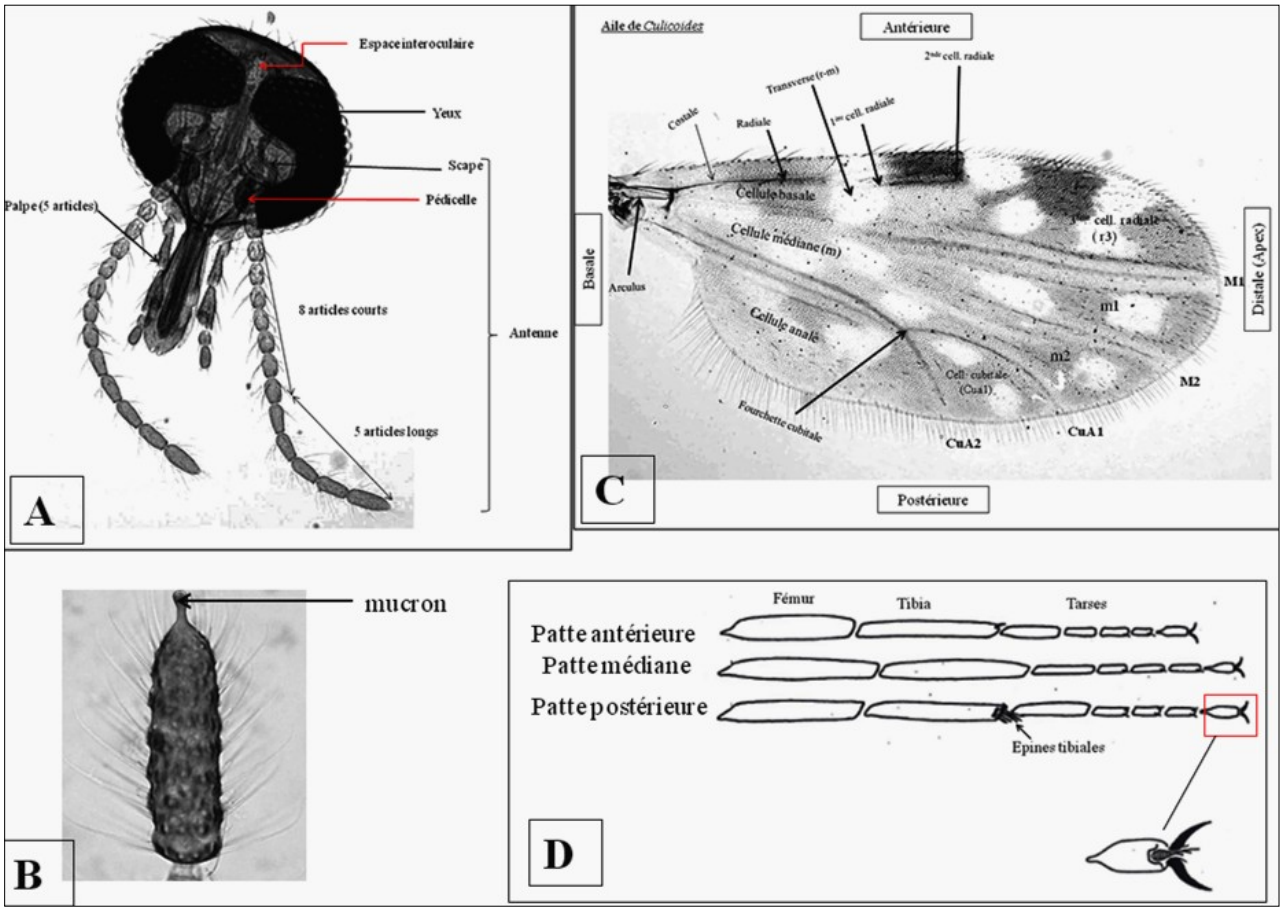

Figure 2 : Caractères morphologiques propres au genre *Culicoides* Latreille. A) tête d'une femelle de *Culicoides* (*C. schultzei* Enderlein, cliché : M.T. Bakhoum, 2017). B) dernier article antennaire d'une femelle de Ceratopogonidae du genre *Forcipomya*, cliché : Jean Claude Delécolle. C) aile de *Culicoides* (*C. schultzei* Enderlein, cliché : M.T. Bakhoum, 2017). D) représentation schématique des pattes de *Culicoides* avec un empodium rudimentaire au niveau du 5ème tarse de la patte postérieure (dessin de Jean-Claude Delécolle, 1985).

2. Diversité et identification à l'espèce du genre *Culicoides* en région Afrotropicale

L'identification à l'espèce repose en premier lieu sur (i) le motif de coloration de l'aile qui varie de sombre avec des taches claires à pale ; dans certains cas, l'aile est sans tache (Figure 3 A) ; et (ii) les yeux soudés ou séparés pouvant être nus ou pubescents (Figure 3 B).

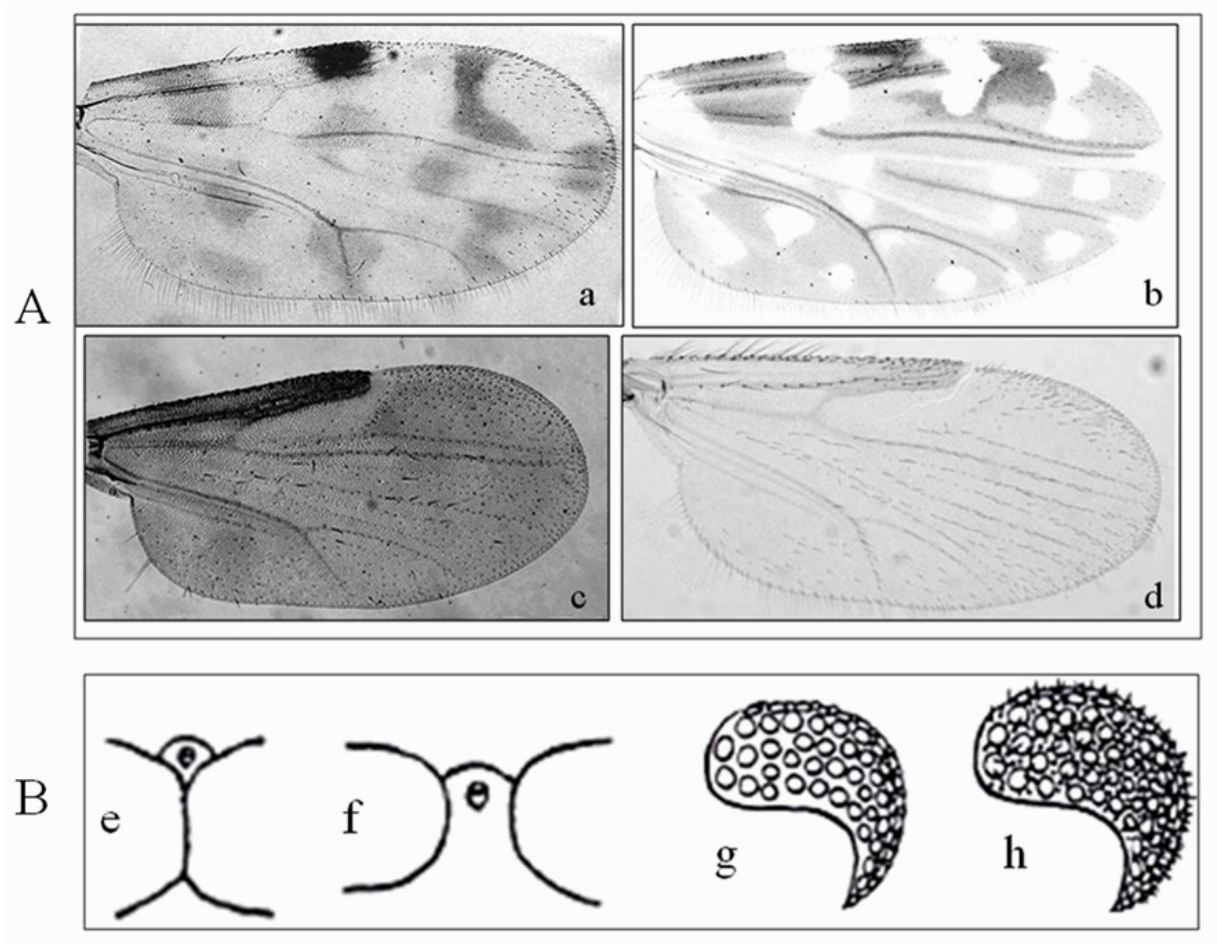

Figure 3 : A) Exemples de motifs d'ailes de femelle de *Culicoides* : a) *C. tuttifrutti* Meiswinkel, Cornet & Dyce ; b) *C. moreli* Clastrier ; c) *C. ravus* De Meillon (clichés : M.T. Bakhoum, 2017) ; d) *C. nigeriae* Ingram & Macfie (cliché : Moussa Fall, 2012). B) Les yeux des espèces de *Culicoides* sont soit e) soudés ou f) séparés pouvant être g) nus ou h) pubescents (dessin de Jean-Claude Delécolle, 1985).

Dans certains cas, une dissection et un montage des spécimens entre lame et lamelle sont nécessaires pour examiner d'autres caractères morphologiques sous un microscope. Ces caractères incluent (i) les sensilles sur les antennes, (ii) la taille et la forme du troisième segment du palpus maxillaire, (iii) le rapport antennaire ($11^{ème}$ article/$10^{ème}$ article), (iv) l'indice antennaire (la longueur des articles longs versus les articles courts), (v) la forme, la taille et la profondeur de la fossette sensorielle ; et (vi) le genitalia. Chez les femelles, on examine le nombre, la forme et la taille des spermathèques (Figure 4), tandis que chez les mâles, l'édéage, les paramères, le $9^{ème}$ sternite et tergite, les processus apicolatéraux et les apodèmes dorsal et ventral du gonocoxite (Figure 5) sont examinés en général (Glick, 1990; Labuschagne, 2016; Wirth et Hubert, 1989).

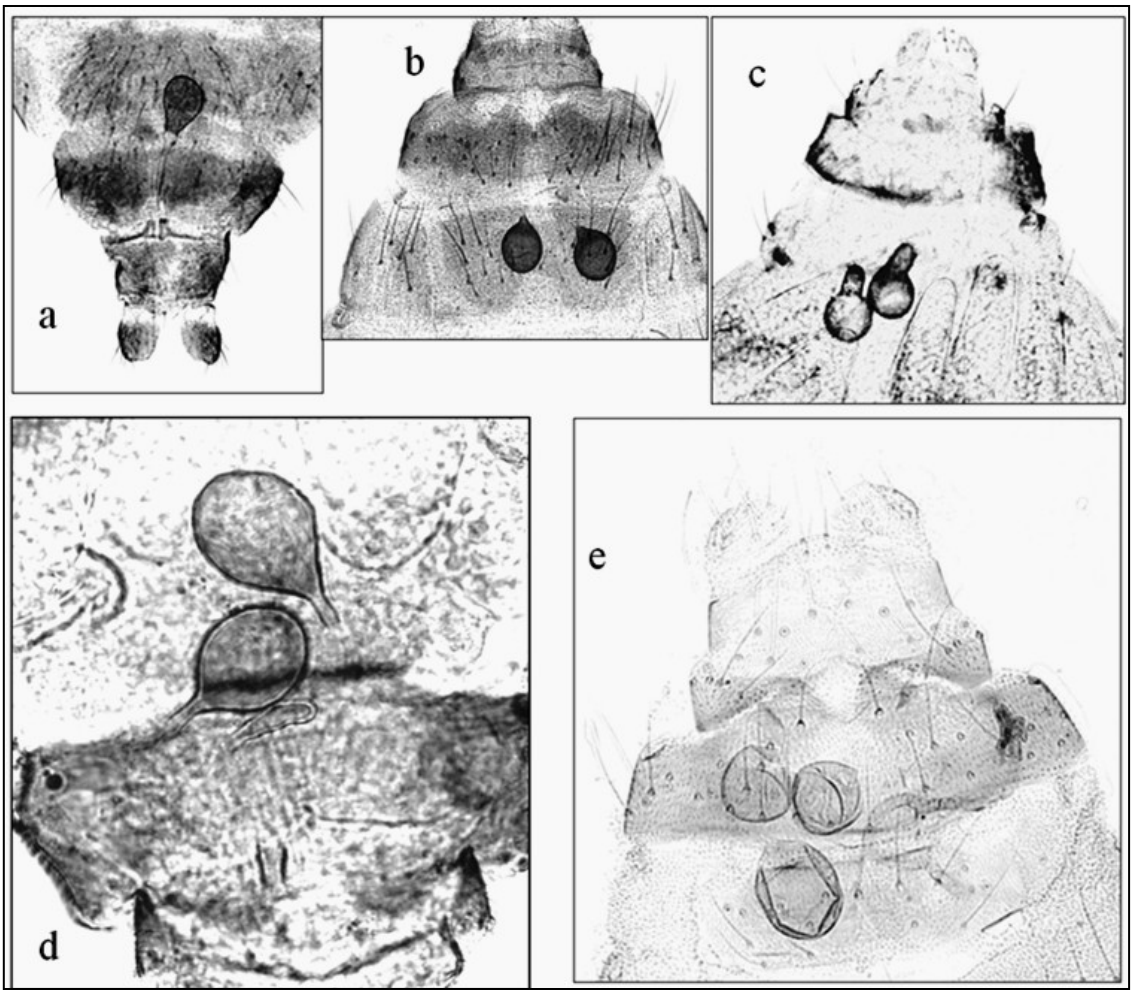

Figure 4 : Exemples de quelques spermathèques servant à la diagnose des femelles de *Culicoides* (clichés : M.T. Bakhoum, 2017) : a) *C. cornutus* De Meillon : une seule spermathèque de forme ovoïde ; b) *C. magnus* Colaco : deux spermathèques arrondis ; c) *C. murphyi* Clastrier & Wirth : deux spermathèques de forme particulière ; d) *C. moucheti* Cornet and Kremer : trois spermathèques dont la 3ème est rudimentaire ; e) *C. tropicalis* Kieffer : trois spermathèques bien développées.

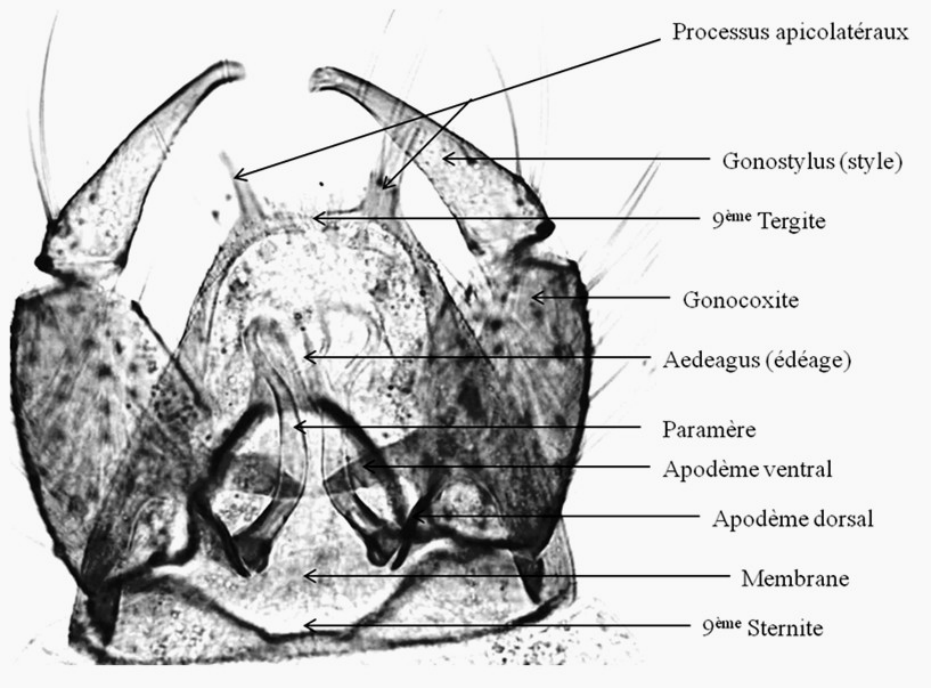

Figure 5 : Genitalia d'un mâle de *C. enderleini* Cornet & Brunhes avec les principales caractéristiques morphologiques utilisées pour la diagnose des mâles de *Culicoides* (cliché : M.T. Bakhoum, 2017).

Entre 1908 et 2015, cent cinquante-six espèces de *Culicoides* ont été décrites en région Afrotropicale (Figure 6).

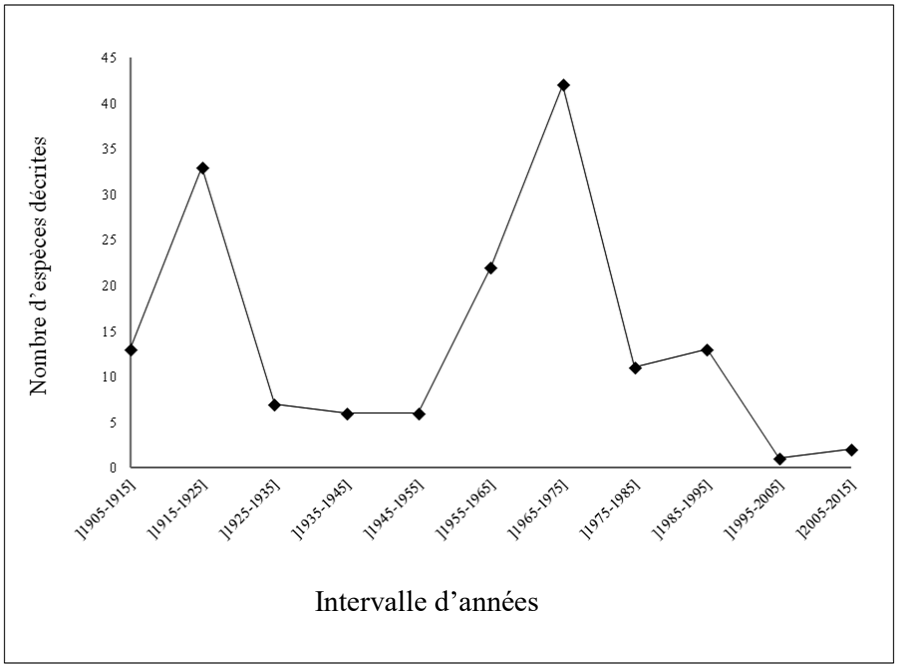

Figure 6 : Nombre d'espèces de *Culicoides* décrites en région Afrotropicale entre 1908 et 2015.

La diversité actuelle est estimée à environ 190 espèces (Bakhoum *et al.*, 2013; Cornet et Chateau, 1970; Cornet *et al.*, 1974; Glick, 1990; Itoua *et al.*, 1987; Labuschagne, 2016; Meiswinkel et Dyce, 1989; Rawlings *et al.*, 2003) réparties dans 9 sous-genres (Tableau 1). Certaines espèces sont groupées mais non classées dans des sous-genres (Tableau 2), d'autres sont non groupées et ne sont affiliées à aucun sous-genre (Tableau 3).

Tableau 1: Liste et distribution géographique des espèces de *Culicoides* classées dans les 9 sous-genres connus en région Afrotropicale

Espèce	Groupe	Sous-genre	Distribution géographique connue	Références
C. candolfii Delécolle et al.	-	*Avaritia*	Gabon	(Delecolle *et al.*, 2013)
C. huambensis Caeiro	-	*Avaritia*	Afrique du Sud, Angola et Zimbabwe	(Labuschagne, 2016; Meiswinkel, 1996)
C. kibatiensis Goetghebuer	-	*Avaritia*	Afrique australe, orientale et centrale	(Labuschagne, 2016; Meiswinkel, 1996)
C. sousadiasi Caeiro	-	*Avaritia*	Angola	(Borkent, 2016)
C. alticola Kieffer	Dasyops	*Avaritia*	Tanzanie	(Meiswinkel, 1987)
C. dasyops Clastrier	Dasyops	*Avaritia*	Sénégal	(Clastrier, 1958; Meiswinkel, 1987)
C. kanagai Khamala and Kettle	Dasyops	*Avaritia*	Afrique australe et orientale	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. glabripennis Goetghebuer	Grahamii	*Avaritia*	Région Afrotropicale (très rare)	(Itoua *et al.*, 1987; Meiswinkel, 1996)
C. grahamii Austen	Grahamii	*Avaritia*	Région Afrotropicale	(Itoua *et al.*, 1987; Meiswinkel, 1996)
C. gulbenkiani Caeiro	Gulbenkiani	*Avaritia*	Afrique du Sud et Côte du Kenya	(Glick, 1990; Meiswinkel, 1996)
C. tororoensis Khamala & Kettle	Gulbenkiani	*Avaritia*	Afrique australe et orientale	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. bolitinos Meiwinkel	Imicola	*Avaritia*	Région Afrotropicale	(Fall *et al.*, 2015a; Meiswinkel, 1996)
C. imicola Kieffer	Imicola	Avaritia	Région Afrotropicale	(Fall *et al.*, 2015a; Glick, 1990; Labuschagne, 2016)
C. loxodontis Meiswinkel	Imicola	Avaritia	Afrique australe et orientale	(Meiswinkel, 1996)
C. miombo Meiswinkel	Imicola	Avaritia	Région Afrotropicale	(Fall *et al.*, 2015a; Labuschagne, 2016)
C. pseudopallidipennis Clastrier	Imicola	Avaritia	Afrique de l'Ouest	(Clastrier, 1958; Fall *et al.*, 2015a; Glick, 1990)
C. tuttifrutti Meiswinkel, Cornet & Dyce	Imicola	Avaritia	Afrique australe	(Labuschagne, 2016; Meiswinkel et Linton, 2003)
C. brosseti Vattier & Adam	Orientalis	Avaritia	Gabon	(Vattier et Adam, 1966)
C. dubitatus Kremer et al.	Orientalis	Avaritia	Angola	(Kremer *et al.*, 1975)
C. trifasciellus Goetghebuer	Orientalis	Avaritia	Afrique centrale et australe	(Labuschagne, 2016; Meiswinkel, 1996)
C. nivosus De Meillon	-	*Beltranmyia*	Largement région Afrotropicale	(Fall *et al.*, 2015a; Glick, 1990; Labuschagne, 2016)
C. pycnostictus Ingram & Macfie	-	*Beltranmyia*	Région Afrotropicale	(Fall *et al.*, 2015a; Glick, 1990; Labuschagne, 2016)
C. brucei Austen	-	*Culicoides*	Région Afrotropicale (très rare)	(Labuschagne, 2016; Meiswinkel, 1996)
C. magnus Colaco	-	*Culicoides*	Afrique du Sud, Angola et Kenya	(Glick, 1990; Itoua *et al.*, 1987; Labuschagne, 2016)
C. sellersi Boorman & Dipeolu	-	*Culicoides*	Nigeria et Sénégal	(Boorman et Dipeolu, 1979; Fall *et al.*, 2015a)
C. sylvicola Khamala & Kettle	-	*Culicoides*	Kenya et Malawi	(Glick, 1990; Khamala et Kettle, 1971; Meiswinkel, 1996)
C. distinctipennis Austen	-	*Meijerehelea*	Région Afrotropicale	(Fall *et al.*, 2015a; Itoua *et al.*, 1987; Labuschagne, 2016)
C. hildae Cornet & Nevill	-	*Meijerehelea*	Afrique du sud	(Labuschagne, 2016; Meiswinkel, 1996)
C. isechnoensis Glick	-	*Meijerehelea*	Kenya	(Glick, 1990; Meiswinkel, 1996)
C. leucostictus Kieffer	-	*Meijerehelea*	Région Afrotropicale	(Meiswinkel, 1996)
C. cornutus De Meillon	Cornutus	*Monoculicoides*	Région Afrotropicale (très rare)	(Itoua *et al.*, 1987; Meiswinkel, 1996)
C. engubandei De Meillon	-	*Pontoculicoides*	Région Afrotropicale (zone humide et très rare)	(Labuschagne, 2016; Meiswinkel, 1996)
C. enderleini Cornet & Brunhes	Schultzei	*Remmia*	Région Afrotropicale	(Bakhoum *et al.*, 2013; Meiswinkel, 1996)
C. kingi Austeni	Schultzei	*Remmia*	Sahel jusqu'au nord du Kenya	(Bakhoum *et al.*, 2013; Cornet et Brunhes, 1994)
C. nevilli Cornet & Brunhes	Schultzei	*Remmia*	Région Afrotropicale	(Bakhoum *et al.*, 2013; Cornet et Brunhes, 1994)
C. oxystoma Kieffer	Schultzei	*Remmia*	Sénégal	(Bakhoum *et al.*, 2013)
C. rhizophorensis Khamala & Kettle	Schultzei	*Remmia*	Afrique orientale et australe	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. schultzei Enderlein	Schultzei	*Remmia*	Afrique orientale et australe (zone aride)	(Meiswinkel, 1996)
C. subschultzei Cornet & Brunhes	Schultzei	*Remmia*	Afrique orientale et australe (zone aride)	(Meiswinkel, 1996)

Tableau 1 (Suite)

Espèce	Groupe	Sous-genre	Distribution géographique connue	Références
C. congolensis Clastrier	Tropicalis	*Synhelea*	Afrique de l'Ouest et centrale	(Meiswinkel, 1996; Meiswinkel et Dyce, 1989)
C. dispar Clastrier	Tropicalis	*Synhelea*	Afrique de l'Ouest	(Meiswinkel, 1996; Meiswinkel et Dyce, 1989)
C. dutoiti De Meillon	Tropicalis	*Synhelea*	Région Afrotropicale (très rare)	(Meiswinkel, 1996; Meiswinkel et Dyce, 1989)
C. moucheti Cornet & Kremer	Tropicalis	*Synhelea*	Afrique de l'Ouest	(Itoua *et al.*, 1987; Meiswinkel et Dyce, 1989)
C. perettii Cornet & Chateau	Tropicalis	*Synhelea*	Région Afrotropicale	(Meiswinkel, 1996; Meiswinkel et Dyce, 1989)
C. tauffliebi Clastrier	Tropicalis	*Synhelea*	Congo	(Itoua *et al.*, 1987; Meiswinkel et Dyce, 1989)
C. tropicalis Kieffer	Tropicalis	*Synhelea*	Région Afrotropicale	(Fall *et al.*, 2015a; Labuschagne, 2016; Meiswinkel, 1996)
C. vicinus Clastrier	Tropicalis	*Synhelea*	Afrique de l'Ouest	(Meiswinkel, 1996; Meiswinkel et Dyce, 1989)
C. fulvithorax Austen	Fulvithorax	*Trithecoides*	Région Afrotropicale (très rare)	(Fall *et al.*, 2015a; Glick, 1990; Labuschagne, 2016)
C. ochrothorax Carter	-	*Trithecoides*	Afrique de l'Ouest et centrale	(Itoua *et al.*, 1987)

Tableau 2 : Liste et distribution géographique des espèces de *Culicoides* classées dans les 11 groupes sans affiliation subgénérique et présentes en région Afrotropicale

Espèce	Groupe	Distribution géographique connue	Références
C. bedfordi Ingram & Macfie	Bedfordi	Afrique orientale et australe (rare)	(Labuschagne, 2016; Meiswinkel, 1996)
C. accraensis Carter, Ingram & Macfie	Accraensis	Région Afrotropicale (très rare)	(Glick, 1990; Meiswinkel, 1996)
C. albopunctatus Clastrier	Accraensis	Congo	(Meiswinkel et Dyce, 1989)
C. olyslageri Kremer & Nevill	Accraensis	Afrique orientale et australe	(Labuschagne, 2016; Meiswinkel, 1996)
C. papillatus Khamala and Kettle	Accraensis	Kenya	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. spinulosus Khamala & Kettle	Accraensis	Kenya	(Meiswinkel, 1996)
C. translucens Khamala & Kettle	Accraensis	Région Afrotropicale (très rare)	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. albovenosus Khamala & Kettle	Albovenosus	Angola, Kenya et Malawi	(Glick, 1990; Meiswinkel, 1996)
C. angolensis Caeiro	Albovenosus	Angola	(Khamala et Kettle, 1971)
C. bernardae Itoua & Cornet	Bernardae	Congo	(Itoua *et al.*, 1987)
C. coarctatus Clastrier & Wirth	Coarctatus	Région Afrotropicale (très rare)	(Labuschagne, 2016; Meiswinkel, 1996)
C. dekeyseri Clastrier	Dekeyseri	Région Afrotropicale	(Labuschagne, 2016; Meiswinkel, 1996)
C. galliardi Callot, Kremer & Molet	Dekeyseri	Lesotho	(Meiswinkel, 1996)
*C. hirsutus*Khamala & Kettle	Dekeyseri	Kenya et Uganda	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. kaimosiensis Khamala & Kettle	Dekeyseri	Kenya	(Meiswinkel, 1996)
C. zikaensis Khamala & Kettle	Dekeyseri	Afrique orientale	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. amaniensis Khamala & Kettle	Inornatipennis	Tanzanie	(Khamala et Kettle, 1971)
C. arenarius Edwads	Inornatipennis	Somalie	(Borkent, 2016)
C. excavatus Khamala & Kettle	Inornatipennis	Kenya et Uganda	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. inornatipennis Carter, Ingram & Macfie	Inornatipennis	Région Afrotropicale	(Meiswinkel, 1996)
C. kumbaensis Callo et al.	Inornatipennis	Cameroun	(Itoua *et al.*, 1987)
C. nairobiensis Glick	Inornatipennis	Afrique du Sud, Kenya et Zimbabwe	(Glick, 1990; Meiswinkel, 1996)
C. nigeriae Ingram & Macfie	Inornatipennis	Afrique du Sud, Nigéria	(Borkent, 2016; Labuschagne, 2016)
C. africanus Clastrier	Milnei	Région Afrotropicale (zone chaude, très rare)	(Borkent, 2016; Meiswinkel, 1996)
C. austeni Carter, Ingram & Macfie	Milnei	Afrique de l'Ouest et Centrale	(Cornet *et al.*, 1974; Itoua *et al.*, 1987)
C. diamouanganai Itoua & Cornet	Milnei	Congo	(Borkent, 2016; Itoua *et al.*, 1987)
C. giganteus Khamala & Kettle	Milnei	Kenya (zones montagneuses)	(Meiswinkel, 1996)
C. hortensis Khamala & Kettle	Milnei	Kenya et Uganda	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. isioloensis Cornet, Nevill and Walker	Milnei	Afrique du Sud et Kenya	(Cornet *et al.*, 1974; Meiswinkel, 1996)
C. kerichoensis Khamala and Kettle	Milnei	Afrique du Sud et Kenya	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. krameri Clastrier	Milnei	Région Afrotropicale (rare)	(Cornet *et al.*, 1974; Meiswinkel, 1996)
C. milnei Austen	Milnei	Région Afrotropicale, (très rare)	(Fall *et al.*, 2015a; Labuschagne, 2016; Meiswinkel, 1996)
C. moreli Clastrier	Milnei	Région Afrotropicale	(Fall *et al.*, 2015a; Labuschagne, 2016; Meiswinkel, 1996)
C. murtalai Boorman & Dipeolu	Milnei	Nigeria	(Boorman et Dipeolu, 1979)
C. nobrei Caeiro	Milnei	Afrique centrale	(Cornet *et al.*, 1974)
C. quinquelineatus Goetghebuer	Milnei	Afrique de l'Ouest et centrale	(Cornet *et al.*, 1974; Meiswinkel, 1996)
C. rutshuruensis Goetghebuer	Milnei	Congo et République démocratique du Congo	(Itoua *et al.*, 1987)
C. trouilleti Itoua & Comet	Milnei	Congo	(Itoua *et al.*, 1987)
C. wansoni Goetghebuer	Milnei	Bassin du Congo	(Cornet *et al.*, 1974)
C. zuluensis De Meillon	Milnei	Afrique australe et orientale	(Labuschagne, 2016; Meiswinkel, 1996)
C. vitshumbiensis Goetghebuer	Milnei	République démocratique du Congo	(Cornet *et al.*, 1974; Meiswinkel, 1996)

Tableau 2 (Suite)

Espèce	Groupe	Distribution géographique connue	Références
C. neavei Austen	Neavei	Région Afrotropicale	(Itoua *et al.*, 1987; Meiswinkel, 1996)
C. ovalis Khamala & Kettle	Neavei	Région Afrotropicale (rare)	(Glick, 1990; Itoua *et al.*, 1987; Meiswinkel, 1996)
C. vomensis Boorman & Dipeolu	Neavei	Afrique de l'Ouest	(Boorman et Dipeolu, 1979)
C. yankari Boorman & Dipeolu	Neavei	Nigeria; Sénégal	(Boorman et Dipeolu, 1979; Fall *et al.*, 2015a)
C. kotonkan Boorman and Dipeolu	Nigripennis	Nigeria	(Meiswinkel, 1996)
C. nigripennis Carter, Ingram & Macfie	Nigripennis	Afrique de l'Ouest et centrale	(Itoua *et al.*, 1987; Meiswinkel, 1996)
C. rageaui Vattier & Adam	Nigripennis	Congo	(Itoua *et al.*, 1987)
C. camicasi Cornet et Château	Similis	Afrique de l'Ouest	(Cornet et Chateau, 1970; Meiswinkel, 1996)
C. corneti Kremer	Similis	Angola	(Itoua *et al.*, 1987)
C. exspectator Clastrier	Similis	Région Afrotropicale (large distribution)	(Itoua *et al.*, 1987; Meiswinkel, 1996)
C. grenieri Vattier & Adam	Similis	Congo	(Itoua *et al.*, 1987)
C. herero Enderlein	Similis	Régions désertiques	(Labuschagne, 2016; Meiswinkel, 1996)
C. karenensis Glick	Similis	Kenya	(Glick, 1990; Meiswinkel, 1996)
C. kobae Cornet and Château	Similis	Région Afrotropicale (zones chaudes et de faible altitude)	(Meiswinkel, 1996)
C. micheli Cornet and Château	Similis	Afrique de l'Ouest	(Cornet et Chateau, 1970)
C. onderstepoortensis Fiedler	Similis	Afrique du Sud	(Labuschagne, 2016; Meiswinkel, 1996)
C. parvulus Khamala and Kettle	Similis	Kenya	(Khamala et Kettle, 1971; Meiswinkel, 1996)
C. pellucidus Khamala and Kettle	Similis	Tanzanie	(Khamala et Kettle, 1971)
C. pretoriensis Kremer & Nevill	Similis	Afrique orientale et australe	(Meiswinkel, 1996)
C. radiomaculatus Khamala & Kettle	Similis	Kenya	(Glick, 1990; Meiswinkel, 1996)
C. ravus De Meillon	Similis	Afrique orientale et australe	(Meiswinkel, 1996)
C. similis Carter, Ingram & Macfie	Similis	Région Afrotropicale	(Fall *et al.*, 2015a; Labuschagne, 2016; Meiswinkel, 1996)

Tableau 3: Liste et distribution géographique de diverses espèces de *Culicoides* non groupées et non classées dans un sous-genre en région Afrotropicale

Espèce	Distribution géographique connue	Références
C. adamskii Wirth	Seychelles	(Borkent, 2016)
C. adersi Ingram & Macfie	Côte du Kenya et Tanzanie	(Glick, 1990; Meiswinkel, 1996)
*C. albosparsus*Kieffer	Ethiopie	(Borkent, 2016)
C. azerbajdzhanicus Dzhafarov	Sénégal	(Fall *et al.*, 2015a)
C. barrosmachadoi Callot, Kremer & Molet	Angola	(Borkent, 2016)
C. bassetorum Callot, Kremer & Molet	Lesotho	(Borkent, 2016)
C. bisignatus Kieffer	Cameroun	(Borkent, 2016)
C. bisolis Kremer & Brunhes	Madagascar	(Borkent, 2016)
C. bwambanus De Meillon	Région Afrotropicale	(Labuschagne, 2016; Meiswinkel, 1996)
C. châteaui Cornet	Sénégal	(Cornet et Chateau, 1970)
C. citroneus Carter, Ingram & Macfie	Région Afrotropicale (zones forestières)	(Labuschagne, 2016; Meiswinkel, 1996)
C. clarkei Carter, Ingram & Macfie	Région Afrotropicale (très rare)	(Labuschagne, 2016; Meiswinkel, 1996)
C. confusus Carter, Ingram & Macfie	Ghana	(Borkent, 2016)
C. corsoni Ingram & Macfie	Ghana	(Borkent, 2016)
C. dentatus Kieffer	Cameroun	(Itoua *et al.*, 1987)
C. eriodendroni Carter, Ingram & Macfie	Région Afrotropicale	(Glick, 1990; Meiswinkel, 1996)
C. gambiae Clastrier	Région Afrotropicale	(Fall *et al.*, 2015a; Labuschagne, 2016; Meiswinkel, 1996)
C. guineensis Kieffer	Guinée	(Borkent, 2016)
C. jouberti Huttel, Huttel and Verdier	Gabon	(Borkent, 2016)
C. korossoensis Huttel and Huttel	Mali	(Borkent, 2016)
C. kribiensis Kieffer	Cameroun	(Itoua *et al.*, 1987)
C. lamborni Ingram & Macfie	Malawi	(Borkent, 2016)
C. macintoshi Cornet and Nevill	Afrique du sud	(Labuschagne, 2016; Meiswinkel, 1996)
C. madagascarensis De Meillon	Madagascar	(Borkent, 2016)
C. murphyi Clastrier & Wirth	Afrique de l'Ouest	(Fall *et al.*, 2015a)
C. nilogenus Kieffer	Soudan	(Borkent, 2016)
C. nilophilus Kieffer	Soudan	(Borkent, 2016)
C. octosignatus Kieffer	République démocratique du Congo	(Itoua *et al.*, 1987)
C. punctithorax Carter, Ingram & Macfie	Région Afrotropicale (rare)	(Labuschagne, 2016; Meiswinkel, 1996)
C. quadrisignatus Kieffer	Cameroun	(Itoua *et al.*, 1987)
C. remerki Boorman & Dipeolu	Région Afrotropicale	(Labuschagne, 2016)
C. remotus Kieffer	Guinée	(Borkent, 2016)
C. robini Cornet	Afrique de l'Ouest et centrale	(Itoua *et al.*, 1987)
C. rutilus Ingram & Macfie	Ghana	(Borkent, 2016)
C. saboyae Cornet	Sénégal	(Cornet et Chateau, 1970)
C. shimoniensis Khamala & Kettle	Afrique du Sud et Kenya	(Labuschagne, 2016; Meiswinkel, 1996)
C. signatus Kieffer	Soudan	(Borkent, 2016)
C. silvestrii Kieffer	Cameroun	(Itoua *et al.*, 1987)
C. stercorarius Khamala & Kettle	Afrique du Sud et Kenya	(Labuschagne, 2016)

Tableau 3 (Suite)

Espèce	Distribution géographique connue	Références
C. taylori Boorman and Lane	Nigeria	(Borkent, 2016)
C. trisignatus Kieffer	Cameroun	(Itoua *et al.*, 1987)
C. tristanii Huttel, Huttel & Verdier	Gabon	(Itoua *et al.*, 1987)
C. walkeri Boorman	Afrique du Sud et Kenya	(Labuschagne, 2016; Meiswinkel, 1996)
C. xanthogaster Kieffer	Guinée	(Borkent, 2016)

3. Etat des connaissances bio-écologiques

Les espèces du genre *Culicoides* ont un développement holométabole, c'est-à-dire que la larve et la nymphe ne ressemblent pas à l'adulte (Figure 7).

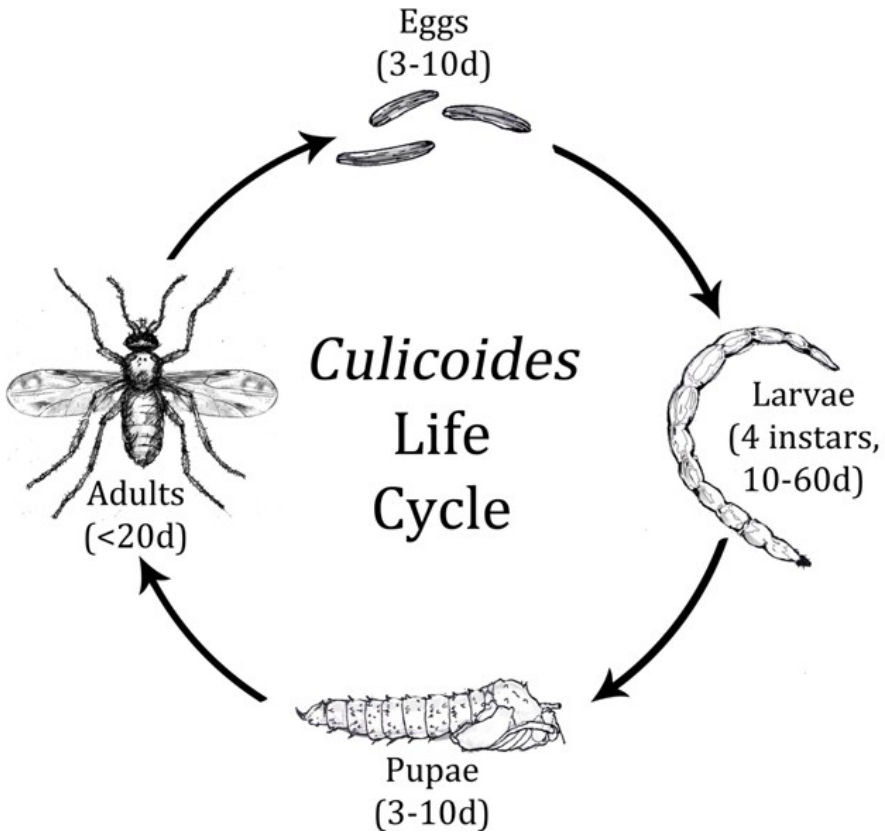

Figure 7 : Cycle de vie du genre *Culicoides*. Image tirée de Purse et al. (2015)

Les *Culicoides* adultes vivent généralement dans des endroits humides et ombragés. En région Paléarctique, la plupart de ces espèces sont au repos dans la végétation durant la journée (Zimmer *et al.*, 2014). Cependant, peu d'études ont été réalisées sur le sujet et il est probable qu'il existe une diversité de sites de repos en fonction des espèces étudiées et des régions. Les femelles sont hématophages (Birley et Boormann, 1982) mais peuvent se nourrir également de jus sucré (Campbell et Kettle, 1975) ; et les mâles sont dits floricoles (Goetghebuer, 1952) et se nourrissent uniquement de sucs de végétaux et de pollen (Chaker, 1983).

La notion de préférence trophique d'une espèce hématophage est par définition, la tendance à sélectionner un type d'hôte dans une classe de vertébrés (Clements, 1999). Le comportement trophique chez les espèces de *Culicoides* est très peu décrit, et étudié à travers des captures sur appât. Les études montrent que les espèces de *Culicoides* en région Afrotropicale se nourrissent sur divers hôtes de mammifères et d'oiseaux (Tableau 4) (Auriault, 1979; El

Sinnary *et al.*, 1985; Fall *et al.*, 2015b; Fall *et al.*, 2015c; Itoua *et al.*, 1987; Labuschagne, 2016).

L'accouplement entre mâles et femelles de *Culicoides* a lieu le plus souvent dans des essaims précédé d'un vol nuptial (Campbell et Kettle, 1979). Cependant, là encore, peu de choses sont connues sur le comportement de reproduction des *Culicoides* (Campbell et Kettle, 1979). Après l'accouplement, le sperme contenu dans les spermatophores des mâles (sacs à sperme) est transféré vers la ou les spermathèque(s) des femelles, en fonction des espèces de *Culicoides*. Une fois fécondées, les femelles partent en quête de leur premier repas de sang nécessaire à la maturation des œufs. La fréquence des repas de sang est de 3 à 5 jours selon la disponibilité des hôtes, période nécessaire pour compléter leur cycle trophogonique (Birley et Boormann, 1982; Braverman, 1988; Holmes et Birley, 1987). Après la maturation des œufs, qui survient 2 à 4 jours après le repas de sang (Zimmer *et al.*, 2014), les femelles cherchent un gîte d'oviposition pour déposer leurs œufs. Le nombre d'œufs pondus varie entre 30 à 450 pour une taille comprise entre 350 et 500 µm de longueur et entre 65 et 80 µm de largeur. De l'œuf éclot une larve vermiforme et dépourvue de pseudopodes (Figure 7) dans les 3 à 10 jours suivant la ponte (Purse *et al.*, 2015). Les larves se nourrissent de débris organiques divers et/ou sont prédatrices de nématodes, bactéries, protozoaires, voire même de leurs propres congénères (Chaker, 1983; Hill, 1947; Kettle, 1962). Les habitats larvaires des espèces du genre *Culicoides* sont essentiellement humides et enrichis en matière organique d'origine animale ou végétale. Ils sont divers et variés : (i) bords des marais, ruisseaux, rivières, plages et piscines ; (ii) pourtours des flaques d'eau et abreuvoirs ; (iii) trous d'arbres et autres cavités de bois ; (iv) fruits en décomposition ; (v) excréments de grands animaux herbivores ; (vi) boue associée à de la litière; et (vii) tas de fumier humide (Blackwell *et al.*, 1994; Braverman *et al.*, 1974; Dipeolu et Ogunrinade, 1976; Gonzalez *et al.*, 2013; Jenkins et Young, 2010; Lubega et Khamala, 1976; Meiswinkel, 1989; Nevill, 1967). En région Afrotropicale, on trouve des habitats larvaires spécifiques pour une espèce d'une part, et d'autre part des espèces de *Culicoides* peuvent partager le même habitat larvaire ou se retrouver dans plusieurs habitats différents (Tableau 5) (Dipeolu et Ogunrinade, 1976; Jenkins et Young, 2010; Labuschagne, 2016; Lubega et Khamala, 1976; Meiswinkel, 1987; Meiswinkel, 1992; Nevill, 1967; Nevill *et al.*, 2007). Il est important de noter que malgré la diversité d'habitats larvaires décrits précédemment, les habitats larvaires de nombreuses espèces restent inconnus dans plusieurs parties du monde.

A priori le développement larvaire est optimal dans des milieux semi-aquatiques et riches en débris organiques (Goetghebuer, 1952; Zimmer *et al.*, 2014), et se fait en 4 stades (Purse *et*

al., 2015). La larve de stade IV devient une nymphe qui reste quasi immobile et munie de cornes respiratoires. L'imago (mâle ou femelle) émerge au bout de 3 à 10 jours. La durée de vie d'un adulte de *Culicoides* est inférieure à 20 jours (Purse *et al.*, 2015) mais peut aller jusqu'à 90 jours en conditions de laboratoire (Boorman, 1991).

Tableau 4 : Association espèce-hôte de 26 espèces de *Culicoides* en région Afrotropicale décrites dans la littérature

***Culicoides* spp.**	**Bovin**	**Caprin**	**Cheval**	**Humain**	**Oiseau**	**Ovin**	**Rongeur**	**Porc**
C. adersi Ingram & Macfie				x				
C. austeni Carter, Ingram & Macfie				x				
C. bedfordi Ingram & Macfie			x					
C. bolitinos Meiwinkel			x			x		
C. brucei Austen	x			x	x			
C. cornutus De Meillon							x	
C. distinctipennis Austen				x				
C. engubandei De Meillon	x		x					
C. fulvithorax Austen			x	x				x
C. grahamii Austen				x				
C. gulbenkiani Caeiro			x			x		
C. imicola Kieffer	x		x		x	x		
C. kingi Austeni	x		x			x		
C. krameri Clastrier	x			x				
C. leucostictus Kieffer					x			
C. magnus Colaco								
C. milnei Austen	x		x	x	x	x		
C. neavei Austen				x	x			
C. nivosus De Meillon					x			
C. oxystoma Kieffer			x			x		
C. pseudopallidipennis Clastrier			x			x		
C. pycnostictus Ingram & Macfie	x		x		x			
C. schultzei Enderlein	x		x					
C. tororoensis Khamala & Kettle		x		x		x		
C. trifasciellus Goetghebuer				x				
C. zuluensis De Meillon		x	x		x	x		

Tableau 5 : Différents types d'habitats larvaires décrits dans la littérature pour 47 espèces de *Culicoides* en région Afrotropicale : (a) bords des marais, rivières, lacs d'eau douce ou salée ; (b) pourtours des flaques d'eau et abreuvoirs ; (c) trous d'arbres et autres cavités de bois ; (d) fruits en décomposition ; (e) excréments de grands animaux herbivores ; (f) boue associée à de la litière; (g) tas de fumier humide

Culicoides spp.	**a**	**b**	**c**	**d**	**e**	**f**	**g**
C. accraensis Carter, Ingram & Macfie		x	x				
C. adersi Ingram & Macfie	x						
C. austeni Carter, Ingram & Macfie		x				x	
C. bedfordi Ingram & Macfie	x	x		x			
C. bolitinos Meiwinkel					x		
C. brucei Austen	x						x
C. clarkei Carter, Ingram & Macfie							
C. confusus Carter, Ingram & Macfie		x					
C. cornutus De Meillon		x					x
C. distinctipennis Austen		x	x				
C. enderleini Cornet & Brunhes							
C. eriodendroni Carter, Ingram & Macfie			x				
C. exspectator Clastrier	x	x					
C. fulvithorax Austen		x		x			
C. gambiae Clastrier	x	x					
C. grahamii Austen	x		x	x			
C. gulbenkiani Caeiro		x			x		
C. imicola Kieffer		x			x		
C. inornatipennis Carter, Ingram & Macfie		x					
C. kanagai Khamala and Kettle					x		
C. kibatiensis Goetghebuer		x					
C. kingi Austeni	x						
C. krameri Clastrier		x		x		x	
C. kwagga					x		
C. leucostictus Kieffer		x		x			
C. loxodontis Meiswinkel					x		
C. magnus Colaco		x					
C. milnei Austen		x	x	x		x	
C. moreli Clastrier	x	x					
C. neavei Austen	x	x					
C. nigripennis Carter, Ingram & Macfie			x				
C. nivosus De Meillon	x	x				x	
C. olyslageri Kremer & Nevill							
C. onderstepoortensis Fiedler					x		
C. ovalis Khamala & Kettle				x			
C. oxystoma Kieffer		x					
C. pycnostictus Ingram & Macfie		x		x		x	
C. ravus De Meillon		x					
C. rhizophorensis Khamala & Kettle	x						
C. schultzei Enderlein	x				x		
C. similis Carter, Ingram & Macfie		x		x			
C. stercorarius Khamala & Kettle					x		
C. tororoensis Khamala & Kettle	x						
C. trifasciellus Goetghebuer	x						
C. tropicalis Kieffer	x	x					
C. tuttifrutti Meiswinkel, Cornet & Dyce				x			
C. zuluensis De Meillon		x			x		

4. Importance en santé publique et vétérinaire

La piqûre douloureuse et prurigineuse provoquée par les *Culicoides* : (i) chez l'homme, gêne les pêcheurs et chasseurs, et constitue un problème majeur dans l'essor du tourisme et les activités de foresterie dans certains pays (Auriault, 1979; Itoua *et al.*, 1987; Mullen, 2009); et (ii) est responsable chez les chevaux d'une réaction allergique nommée la dermite estivale récidivante (Kleider et Lees 1984).

Outre la nuisance, certaines espèces du genre *Culicoides* sont impliquées dans la transmission de pathogènes d'intérêt médical et vétérinaire. Un certain nombre d'espèces du genre *Culicoides* en région Afrotropicale sont vectrices avérées ou suspectées de nématodes (Agbolade *et al.*, 2006; Bassene *et al.*, 2015; Debrah *et al.*, 2017; El Sinnary et Hussein, 1980; Simonsen *et al.*, 2011; Stensgaard *et al.*, 2016) (Tableau 6) mais surtout de virus. On estime qu'il y a plus de 50 virus isolés à partir de *Culicoides* capturés sur le terrain à travers le monde (Mellor *et al.*, 2000) dont la plupart appartenant aux familles des Bunyaviridae, Reoviridae et Rhabdoviridae (Mellor *et al.*, 2000; Mullen, 2009). En région Afrotropicale, les espèces de *Culicoides* sont principalement associées à trois virus d'intérêt vétérinaire (Tableau 7, Figure 8), les virus de la fièvre catarrhale ovine, de la fièvre hémorragique épizootique et de la peste équine (Carpenter *et al.*, 2017; Purse *et al.*, 2015).

Tableau 6: Nématodes transmis par des *Culicoides* en région Afrotropicale aux humains et aux animaux domestiques.

Nématode	**Hôte vertébré**	**Zone géographique connue**	**Vecteur connu ou suspecté**
Mansonella perstans	Humain	Afrique subsaharienne	*C. austeni*, *C. fulvithorax*, *C. grahamii*, *C. inornatipennis*, *C.* spp.
Mansonella streptocera	Humain	Afrique de l'Ouest et centrale	*C. austeni*, *C. grahamii*
Onchocerca guttarosa	Bovin	Soudan	*C. kingi*

Tableau 7: Trois arbovirus d'intérêt vétérinaire majeur transmis par des *Culicoides* en région Afrotropicale : Peste équine (PE), Fièvre catarrhale ovine (FCO) et Fièvre hémorragique épizootique (FHE)

Virus	**Hôte vertébré**	**Distribution géographique**	**Vecteur avéré ou suspecté**
PE	Equidés sauvages et domestiques (chevaux, ânes et mulets)	Afrique du Sud, Ethiopie, Namibie et Sénégal	*C. bolitinos*, *C. brucei*, *C. imicola*, *C. oxystoma*
FCO	Ruminants sauvages (cervidés) et domestiques (bovins, ovins et caprins)	Afrique du Sud, Burkina Faso, Kenya, La Reunion, Sénégal et Soudan	*C. bolitinos*, *C. imicola*, *C. exspectator*, *C. milnei*, *C. oxystoma*, *C. pycnostictus*, *C. tororoensis*
FHE	Ruminants sauvages (cervidés) et domestiques (bovins, ovins et caprins)	Afrique du Sud, La Reunion et Nigéria	*C. cornutus*, *C. kingi*, *C. nevilli*, *C. schultzei*

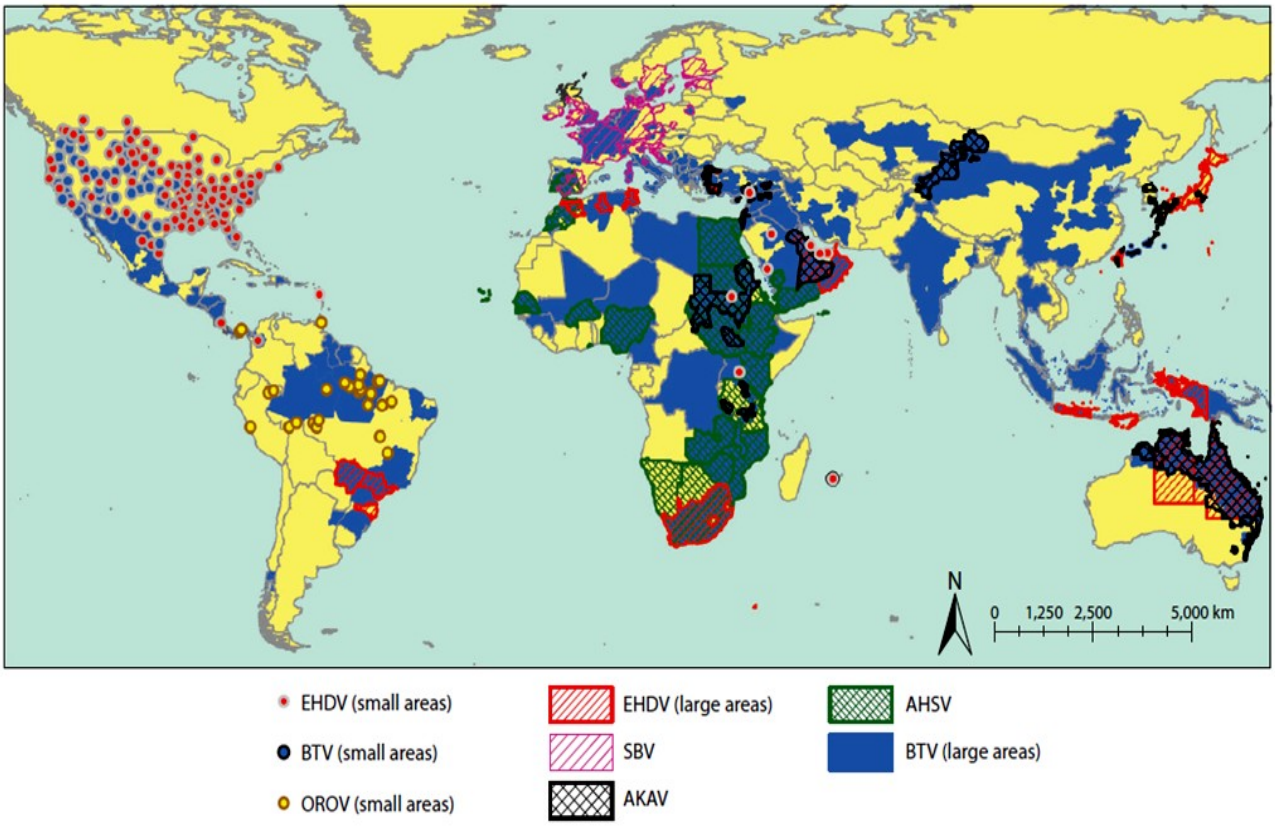

Figure 8 : Distribution de six arbovirus transmis par des espèces du genre *Culicoides*. Image tirée de Purse et al. (2015)

1. La peste équine ou *African Horse Sickness* (AHS)

La peste équine est une maladie virale infectieuse mais non contagieuse, affectant les équidés sauvages (zèbres) et domestiques (chevaux, les ânes et les mulets). La maladie a été reconnue pour la première fois en Afrique du Sud au début des années 1700, et l'agent étiologique isolé plus tard en 1899 à partir de chevaux infectés (Meiswinkel *et al.*, 2004b). Maladie à déclaration obligatoire à l'organisation mondiale de la Santé Animale (OIE), la peste équine est létale chez les chevaux avec plus de 95% de mortalité chez les races sensibles (Mullen, 2009).

Le virus de la peste équine est transmis par certaines espèces de *Culicoides* (Tableau 7). Elle se rencontre dans toute l'Afrique subsaharienne et dans la péninsule Arabique, et a pu s'étendre de façon intermittente dans l'Asie du Sud-ouest, en Afrique du Nord (Maghreb) et en Europe méridionale (Figure 8). L'agent étiologique de la peste équine est un *Orbivirus* de la famille des Reoviridae, étroitement lié aux virus responsables de la fièvre catarrhale ovine et de la maladie hémorragique épizootique. Neuf sérotypes sont reconnus responsables de cette

maladie. Tous les neuf sont présents en Afrique orientale et australe (Meiswinkel *et al.*, 2004b). Seuls les sérotypes 4, 9 et récemment 2 sont signalés en Afrique de l'Ouest (Diouf *et al.*, 2012; Mullen, 2009). La circulation de sérotypes multiples dans une région géographique donnée et les infections simultanées d'animaux avec plus d'un sérotype soulignent la complexité épidémiologique de cette maladie (Mullen, 2009). L'incubation du virus de la peste équine est de durée variable de 3 à 14 jours selon le sérotype et la réceptivité des chevaux sensibles. La maladie se manifeste sous quatre formes (Leforban *et al.*, 1983; Mullen, 2009): (i) la forme pulmonaire est la plus fatale avec un taux de mortalité très élevé, environ 95% ; (ii) la forme cardiaque est caractérisée au début par une fièvre et la congestion des muqueuses et après une période d'incubation de 7 à 14 jours, le taux de mortalité peut atteindre 50% ; (iii) la forme mixte pulmonaire-cardiaque est caractérisée par des signes cliniques associés aux formes précédentes avec la mort qui s'ensuit 3 à 6 jours plus tard, le taux de mortalité est proche de 80% ; et enfin (iv) la forme fébrile ou *horse sickness fever* qui est caractérisée par une hyperthermie accompagnée d'une légère polypnée et d'une tachycardie, moins grave et qui disparaît en général au bout de 15 jours.

2. La fièvre catarrhale ovine ou *Bluetongue disease* (BT)

Comme la peste équine, la fièvre catarrhale ovine est causée par un *Orbivirus* de la famille des Reoviridae (Maclachlan *et al.*, 2009) transmis par certaines espèces de *Culicoides* (Tableau 7). Cette maladie virale non contagieuse affecte les ruminants sauvages et domestiques, notamment les bovins et ovins. La fièvre catarrhale ovine a été reconnue pour la première fois en Afrique du Sud vers les années 1930 suite à l'introduction des moutons de race européenne (Meiswinkel *et al.*, 2004b; Mullen, 2009). Historiquement, cette maladie a été décrite dans les régions tropicales et sub-tropicales entre les 40°-50°N et 35°S (Mullen, 2009; Sellers, 1984), mais depuis le début des années 2000, le virus s'est propagé vers le nord dans certaines parties du bassin méditerranéen et est apparu en Europe du Nord (Figure 8) (Baldet *et al.*, 2005; Mellor et Wittmann, 2002; Purse *et al.*, 2015). La fièvre catarrhale ovine présente un complexe antigénique avec 27 sérotypes connus (Jenckel *et al.*, 2015) qui varient considérablement dans leur pathogénicité (Maclachlan *et al.*, 2009). En région Afrotropicale, une sous-évaluation de la présence du virus est observée du fait de l'absence de signes cliniques chez les races locales de bovins et petits ruminants.

3. La fièvre hémorragique épizootique ou *Epizootic Hemorrhagic Disease* (EHD)

La fièvre hémorragique épizootique est très similaire à la fièvre catarrhale ovine sur plusieurs aspects. Le virus est transmis également par différentes espèces de *Culicoides* (Tableau 7), la différence majeure est que la fièvre hémorragique épizootique affecte principalement les ruminants sauvages, notamment les cerfs. L'agent étiologique est un *Orbivirus* très proche de celui de la fièvre catarrhale ovine, avec 10 sérotypes reconnus dans le monde et répartis en Afrique du Sud, Amérique du Nord, Australie, La Réunion, Nigéria et Japon (Figure 8) (Anthony *et al.*, 2009; Aradaib *et al.*, 1998; Mullen, 2009; Paweska *et al.*, 2005). L'isolation et l'identification de l'agent étiologique est nécessaire pour déterminer avec certitude le virus impliqué.

5. Imbroglio taxonomique et systématique

La systématique et la taxonomie du genre *Culicoides* restent problématiques. Les groupes et sous-genres sont pour la plupart définis sur des ressemblances morphologiques, dont on ne sait si elles sont analogues ou homologues, ou sur la distribution géographique des espèces (Borkent, 2016).

En région Afrotropicale, avant 1970, seulement deux clés d'identification morphologiques étaient disponibles pour les *Culicoides* adultes, essentiellement limitées à des espèces présentes en Afrique australe et orientale (Colçao, 1946; Fiedler, 1951). Les travaux taxonomiques de Khamala & Kettle (1971) en région Afrotropicale est avec la révision anglophone de Glick (1990) en collaboration avec Michel Cornet, spécialiste de la faune ouest-africaine, ont abouti à une clé d'identification morphologique pour les femelles et les mâles adultes de 55 espèces (Glick, 1990; Khamala et Kettle, 1971). En Afrique de l'Ouest et Centrale, les études taxonomiques sont éparses et souvent limitées à des groupes d'espèces (Boorman et Dipeolu, 1979; Cornet et Brunhes, 1994; Cornet et Chateau, 1970; Cornet *et al.*, 1974; Itoua *et al.*, 1987). Certains de ces travaux ont abouti à des clés d'identification dont les monographies et les catalogues sont parfois imprécis, avec des illustrations de qualité moyenne et utilisant des synonymes d'espèces valides pour cette région. L'expertise de Meiswinkel a donné un nouveau regard au genre *Culicoides* en région australe en particulier (Meiswinkel, 1987; 1989; 1991; 1992; 1996; Meiswinkel et Linton, 2003) et récemment la révision taxonomique et systématique de Labuschagne sur la faune sud-africaine a abouti à une clé d'identification de référence des espèces adultes de *Culicoides* pour cette région (Labuschagne, 2016). Entre 2012 et 2015, suite aux épizooties de peste équine de 2007,

d'importantes études taxonomiques et de préférences trophiques ont été réalisées au Sénégal sur les *Culicoides* adultes (Bakhoum *et al.*, 2013; Fall *et al.*, 2015a; Fall *et al.*, 2015b; Fall *et al.*, 2015c). Les révisions taxonomiques et systématiques du genre *Culicoides* de ces deux zones de la région Afrotropicale ont permis de mettre à jour la diversité culicoidienne. Mais dans certains cas, les variations morphologiques intra-spécifiques observées empêchent une bonne délimitation des espèces. A ceux-ci s'ajoute le désaccord des auteurs sur le rattachement de certaines espèces aux groupes et sous-genres. Les espèces apparentées à *C. milnei* Austen sont classées dans le sous-genre *Hoffmania* Fox par Meiswinkel (1996), par opposition à Borkent (Borkent, 2016) et Labuschagne (2016) qui les classent dans le groupe Milnei sans affiliation à un sous-genre. Autre exemple, le groupe Similis, défini par Cornet et Château (1970) incluant toutes les espèces morphologiquement similaires à *C. similis* Carter, Ingram & Macfie, a été révisé par Meiswinkel & Dyce (1989) pour lesquels certaines espèces du groupe Similis ont été affiliées au groupe Tropicalis. Ces derniers auteurs ont ainsi classé les espèces du groupe Tropicalis dans le sous-genre *Synhelea* Kieffier avec comme type *C. tropicalis* Kieffer ; et les espèces du groupe Similis sans sous-genre. Si cet avis est soutenu par Karien Labuschagne (2016), tel n'est pas le cas pour Borkent qui classe les espèces des groupes Similis et Tropicalis dans le sous-genre *Synhelea* (Borkent, 2016).

En outre, l'étude de l'écologie des stades immatures des espèces d'intérêt reste un champ peu exploré du fait notamment du manque d'outils d'aide à l'identification morphologique de ces différents stades. En Afrique du Sud, des caractères morphologiques ont été examinés sur des stades immatures (nymphes) de *Culicoides* visant à l'identification des spécimens appartenant aux groupes Imicola et Similis (Nevill et Dyce, 1994; Nevill *et al.*, 2009; Nevill *et al.*, 2007).

Problématique et objectifs

Contrairement à l'Afrique du Sud et au Sénégal, il y a un manque considérable d'études ou de révisions taxonomiques et systématiques du genre *Culicoides* en région Afrotropicale. Aujourd'hui, un regain d'intérêt apparait pour les *Culicoides* et les pathogènes qu'ils transmettent dans la région Afrotropicale (Agbolade *et al.*, 2006; Purse *et al.*, 2015; Simonsen *et al.*, 2011), mais les variations morphologiques observées entre les individus d'une même espèce lors de la diagnose et le manque ou l'imprécision d'informations sur les espèces types, empêchent une bonne caractérisation et délimitation des espèces. Pour être

identifié avec précision, un spécimen collecté sur le terrain doit être comparé aux spécimens types (holotypes, isotypes, lectotypes, néotypes, épitypes, paratypes…). Malheureusement, les spécimens types des différentes espèces de *Culicoides* sont éparpillés dans divers laboratoires ou musées souvent situés en dehors de la région Afrotropicale, difficiles d'accès ou perdus pour la science. Dans la littérature, les descriptions de ces spécimens sont souvent faites et publiées dans la langue maternelle de l'auteur ce qui complique la compréhension des informations. Les enjeux de la diversité du genre *Culicoides* en région Afrotropicale nécessite d'un nouveau regard taxonomique pour une meilleure identification et caractérisation des espèces de *Culicoides* et notamment celles d'intérêt médical et vétérinaire.

Le challenge est donc d'intégrer la taxonomie moléculaire (phylogénie et délimitation avec différents marqueurs moléculaires) avec celle basée sur la morphologie (taxonomie classique) afin de répondre aux imbroglios taxonomiques observés. Dans ce cadre, nous avons choisi de nous intéresser aux espèces de la région Afrotropicale du sous-genres *Avaritia* Fox ; du sous-genre *Remmia* Glukhova ; du sous-genre *Synhelea* Kieffer ; du groupe Milnei ; du groupe Neavei et du groupe Similis comme modèles d'étude car : (i) beaucoup de ces espèces sont des vectrices avérées ou suspectées de pathogènes d'intérêt sanitaire et économique pour les ruminants et les équins, et il semble qu'il existe pour ces espèces des complexes d'espèces jumelles, (ii) de nombreux variants morphologiques, (iii) des groupes monophylétiques à confirmer ou infirmer, et (iv) enfin l'hypothèse à vérifier que le groupe Similis appartient au sous-genre *Synhelea* (**Chapitre 2**).

Les foyers récents de peste équine au Sénégal (Akakpo *et al.*, 2011; Diouf *et al.*, 2012) ont réitéré un regain d'intérêt pour le genre *Culicoides* dans ce pays (Bakhoum *et al.*, 2013; Diarra *et al.*, 2014; Diarra *et al.*, 2015; Fall *et al.*, 2015a; Fall *et al.*, 2015b; Fall *et al.*, 2015c). Les études du genre *Culicoides* conduites récemment au Sénégal ont été faites sur les populations adultes. A une échelle locale, je me suis intéressé au comportement trophique des espèces vectrices présentes au Sénégal afin de mieux comprendre les interactions hôtes-vecteurs et les préférences trophiques. J'ai adopté une méthodologie nouvelle utilisée en agronomie pour décrire les préférences trophiques de *C. imicola* Kieffer, *C. kingi* Austen et *C. oxystoma* Kieffer en fonction de la distribution des hôtes mammifères potentiels présents sur le site d'étude (**Chapitre 3**).

De plus, je me suis intéressé à investiguer et décrire les habitats larvaires en particulier ceux des espèces d'intérêt en santé vétérinaire (**Chapitre 4).** En outre j'ai utilisé un outil d'aide à l'identification moléculaire des stades immatures, qui permet de s'affranchir de l'absence de clés d'identification morphologique pour les larves ou les pupes de *Culicoides* en région

Afrotropicale. Ce travail participera à la cartographie de la distribution spatiale des espèces d'intérêt vétérinaire intégrant les connaissances sur l'écologie larvaire et de suggérer des méthodes de prévention et de lutte dirigée contre les stades immatures de *Culicoides*.

L'ensemble de ces résultats sont discutés dans une dernière partie, suivie d'une conclusion.

Chapitre 2. Révision taxonomique et systématique des sous-genres *Avaritia* Fox, *Remmia* Glukhova, et des groupes Milnei, Neavei et Similis

Dans le chapitre précédent, nous avons réalisé une revue des connaissances taxonomiques et bio-écologiques du genre *Culicoides* en région Afrotropicale. Nous avons évoqué l'importance vétérinaire et médicale du genre *Culicoides*, ainsi que l'imbroglio systématique et taxonomique du genre dans la région Afrotropicale. Environ 1 400 espèces de *Culicoides* sont décrites à travers le monde (Borkent, 2016). En région Afrotropicale, la diversité atteindrait 190 espèces (Bakhoum *et al.*, 2013; Cornet et Chateau, 1970; Cornet *et al.*, 1974; Glick, 1990; Labuschagne, 2016; Meiswinkel, 1996), avec un inventaire le plus à jour en Afrique du Sud qui comptabilise environ 100 espèces. Les espèces présentes en région Afrotropicale sont classées dans 9 sous-genres (*Avaritia*, *Beltranmyia*, *Culicoides*, *Meijerehelea*, *Monoculicoides*, *Pontoculicoides*, *Remmia*, *Synhelea* et *Trithecoides*), 9 groupes d'espèces sans affiliation infra générique (Accraensis, Albovenosus, Bedfordi, Dekeyseri, Inornatipennis, Milnei, Neavei, Nigripennis et Similis). En revanche, 28% de la faune Afrotropicale n'est affilié à aucun sous-genre et groupe d'espèces. La classification infra générique des espèces du genre *Culicoides* repose sur la similarité de certains caractères morphologiques comme par exemple la forme des ailes ou la structure du genitalia mâle, ce qui ne reflètent pas les vraies relations phylogénétiques (Borkent, 2016). Avec le développement des approches moléculaires, plusieurs marqueurs ont été utilisés pour réviser le schéma systématique actuel du genre et reconstruire les relations phylogénétiques au sein des sous-genres ou entre les groupes (Harrup *et al.*, 2015).

Dans ce chapitre, nous présentons une étude de phylogénie moléculaire avec deux objectifs. Le premier est de réviser la systématique et la taxonomie des *Culicoides* appartenant aux sous-genres *Avaritia*, *Remmia*, *Synhelea* et groupes Milnei, Neavei et Similis avec une approche pour la première fois multi-marqueurs (sous-unité I du cytochrome oxydase (COI), ADN ribosomal 16S, ADN ribosomal 28S et le carbamoyl-phosphate synthetase 2, aspartate transcarbamylase et dihydroorotase (CAD)). Le deuxième objectif est de combiner les données morphologiques et moléculaires afin de redéfinir les limites d'espèces. Les spécimens de *Culicoides* ont été collectés dans différents pays de la région Afrotropicale (Afrique du Sud, Bénin, Burkina Faso, Cameroun, Ethiopie, Ile de la Réunion, Kenya, Madagascar, Mali, Mozambique, Sénégal et Zimbabwe) et au Liban pour *C. oxystoma*, sous-

genre *Remmia*. Les résultats issus de cette étude font l'objet de l'article 1 soumis au journal *Systematic Entomology* le 19 Mai 2017.

Article 1: Bakhoum M.T., Labuschagne K., Huber K., Fall M., Mathieu B., Venter G., Gardès L., Baldet T., Bouyer J., Fall GA., Gimonneau G., Garros C. (**Submitted to *Systematic Entomol*ogy**) Phylogenetic relationships and molecular delimitation of *Culicoides* Latreille (Diptera: Ceratopogonidae) species in the Afrotropical region: interest for the *Avaritia* subgenus

Phylogenetic relationships and molecular delimitation of *Culicoides* Latreille (Diptera: Ceratopogonidae) species in the Afrotropical region: interest for the *Avaritia* subgenus

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Abstract

Phylogenetic relationships of *Culicoides* species of the Afrotropical region are problematic as different authors disagree on the placement of species into specific subgenera or groups. In this study we sequenced two mitochondrial (COI and 16S rDNA) and two nuclear (CAD and 28S rDNA) gene fragments to reconstruct phylogenetic relationships within the *Avaritia, Remmia* and *Synhelea* subgenera and the Milnei, Neavei and Similis groups of *Culicoides* using both Bayesian inference and maximum likelihood approaches. Based on phylogenetic trees we used the bGMYC (Bayesian General Mixed Yule Coalescent model) and the PTP (Bayesian Poisson Tree Processes) to investigate species boundaries. All species relationships within the studied subgenera and groups were well supported by using morphological characters and molecular analyzes. *Avaritia* Fox subgenus includes (i) all the species of the Imicola group, as well as the putative new species, *C. sp*. #22, and we confirmed the monophyly of this group; (ii) the Dasyops group includes *C. kanagai* and *C. sp*. #54 Meiswinkel (new species), shown to be monophyletic; (iii) the *C. sp*. #20 belongs to the Orientalis group; (iv) *C. grahamii*, *C. gulbenkiani* and *C. kibatiensis*. Our results also show that *Remmia* Glukhova subgenus is monophyletic. Relationships of species of the Milnei group were well supported and demonstrate the monophyly of this group. Borkent's classification for Similis group is confirmed.

Key words: *Culicoides*, Afrotropical, phylogeny, taxonomy, bGMYC, Poisson Tree Process,

Introduction

The biting midges in the genus *Culicoides* (Diptera: Ceratopogonidae), transmit a number of viruses to domestic and wild ruminants, and equids (Mellor *et al.*, 2000; Mullen, 2009). This genus is distributed worldwide and includes about 1, 358 described species (Borkent, 2016). Currently this genus is classified into 32 subgenera containing a number of groups; 38 groups are unaffiliated with a subgenus (Borkent, 2016). Moreover there is a long list of miscellaneous species, not placed in any group, representing 13% of the world fauna.

The internal classification of the genus is based on the morphological similarity between species that includes wing pattern or the shape of male genital structures, which in no way reflects real phylogenetic relationships (Borkent, 2016). There is no consensus on the definition of groups or species complexes for the genus *Culicoides*, to the point that the literature is full of identical names for different sets and different names for identical sets (Harrup *et al.*, 2015). The monophyly of subgenera and species groups has been limitedly tested and subgenera require a systematic revision at the global level (Mathieu, 2011). The subgeneric classification is almost phenetic and mostly based on regional assessments, with limited or not updated description of subgeneric definition. Indeed, some subgenera show evidence of being polyphyletic such as the subgenus *Oecacta* (Szadziewski *et al.*, 2016) which is commonly called the "dumping subgenus".

With the advances made in molecular DNA sequencing, several molecular markers were assessed positively for inferring phylogenetic relationships or molecular delineation of *Culicoides* species (Harrup *et al.*, 2015). These tools are now largely used for species identification, especially for those that are difficult to separate morphologically (Pagès & Sarto I Monteys, 2005; Nolan *et al.*, 2007; Pagès *et al.*, 2009; Monaco *et al.*, 2010; Sebastiani *et al.*, 2001) but few used them to assess phylogenetic relationships. Recently, Bellis et al. (2013) published a revision of the *Culicoides* Imicola group using a combination of morphological and molecular analyses. However, the validity of all species groups and subgenera within the systematic classification need to be revised to assess their phylogenetic validity. As an attempt to clarify and help subgeneric affiliation, Harrup et al. (2015) published a wing atlas for the described subgenera. Recently, the classification of the subgenus *Avaritia* was revised using molecular phylogeny (Mathieu, 2011; Bellis *et al.*, 2013; Gopurenko *et al.*, 2015). The critical issue in *Culicoides* systematics is that it requires phylogenetic validity (Harrup *et al.*, 2015; Labuschagne, 2016).

Culicoides-borne pathogens (Diptera: Ceratopogonidae) in the Afrotropical region are of interest because of major recent outbreaks affecting livestock (Mellor *et al.*, 2000; Mullen, 2009; Purse *et al.*, 2015; Carpenter *et al.*, 2017) or human populations (Agbolade *et al.*, 2006; Simonsen *et al.*, 2011; Bassene *et al.*, 2015; Debrah *et al.*, 2017). For example in the last twenty years, African horse sickness outbreaks have been recorded in South Africa (Venter *et al.*, 2006a), Senegal (Diouf *et al.*, 2012) and Namibia (Scacchia *et al.*, 2009). Recently in West and Central Africa, high prevalence rates of *Mansonella perstans* were recorded in *Culicoides* specimens and human populations (Simonsen *et al.*, 2011; Bassene *et al.*, 2015; Debrah *et al.*, 2017). However, there have been few studies on the *Culicoides* fauna of the Afrotropical region. Although the first *Culicoides* in this region was described over a century ago (Enderlein, 1908), despite the high and undoubtedly underestimated diversity of *Culicoides*. Today, *Culicoides* species diversity in the Afrotropical region reaches 190 described species (Cornet & Chateau, 1970; Cornet *et al.*, 1974; Itoua *et al.*, 1987; Meiswinkel & Dyce, 1989; Glick, 1990; Bakhoum *et al.*, 2013; Labuschagne, 2016) with about 105 *Culicoides* species recorded in South Africa (Labuschagne, 2016), and 41 species in Senegal (Fall *et al.*, 2015). These *Culicoides* species are placed in 9 subgenera (*Avaritia*, *Beltranmyia*, *Culicoides*, *Meijerehelea*, *Monoculicoides*, *Pontoculicoides*, *Remmia*, *Synhelea*, and *Trithecoides*); 9 species groups, unplaced to subgenus (Accraensis, Albovenosus, Bedfordi, Dekeyseri, Inornatipennis, Milnei, Neavei, Nigripennis, and Similis) and miscellaneous species, not placed in any group, representing 28% of the Afrotropical fauna. Borkent placed species of Accraensis, Bedfordi and Similis groups within the subgenus *Synhelea* (Borkent, 2016); in contradiction with Meiswinkel and Dyce who published a study of this subgenus only containing the Tropicalis group (Meiswinkel & Dyce, 1989). The Milnei group contains species of medical and veterinary interest as *C. zuluensis* transmit Lesetele virus, bluetongue and Akabane viruses have been isolated from *C. milnei* and *Onchocerca gutturosa* isolated from *C. krameri* (Meiswinkel *et al.*, 2004). This species group is unplaced in any subgenus (Borkent, 2016), whereas Meiswinkel places this group within the subgenus *Hoffmania* (Meiswinkel, 1996). The classification of *Culicoides* species is problematic as different authors disagree on the placement of species into specific subgenera or groups (Khamala & Kettle, 1971; Boorman & Dipeolu, 1979; Itoua *et al.*, 1987; Glick, 1990).

In addition, monographs and catalogues used to identify *Culicoides* species for this region are old, at times inaccurate, with low quality illustrations. Before 1970, only two taxonomic keys were available for adults, mainly limited to the species present in South Africa and East

Africa (Kenya, Tanzania and Uganda) (Colçao, 1946; Fiedler, 1951). The revision undertaken by Glick (1990) in collaboration with Cornet, a West African fauna specialist, includes morphological keys for adult females and males of 55 species identified in Kenya; this remains a vital reference work. In West Africa, taxonomic studies are rare and often limited to sub regions. Cornet and their associates worked on groups of interest such as the Schultzei, Milnei and Similis groups (Cornet & Chateau, 1970; Cornet *et al.*, 1974; Itoua *et al.*, 1987; Cornet & Brunhes, 1994). Some of their works include taxonomic identification keys for adults. Meiswinkel described or re-described and compiled an identification key for adults and immature stages for the species of the *Culicoides imicola* group (Meiswinkel, 1995; Nevill *et al.*, 2007). Therefore, until all species have been compared molecularly as well as morphologically it remains up to individual authors to either use the current published subgeneric classification by Borkent or the species groups (Labuschagne, 2016).

Besides the problematic systematics, species delimitation is also complicated by large morphological variations observed within certain species. Species identification is made more difficult when the name-bearing specimen type is lost, and the description is old with limited drawings or pictures. To overcome this problem, numerous studies have investigated phylogenetic relationships using molecular data together with species delimitation methods in other insect genera (Toussaint *et al.*, 2015). In our opinion, these methods can be used to revise the limit and classification of *Culicoides* species in the Afrotropical region.

In this study, we aimed at (i) inferring phylogenetic relationships of *Culicoides* species collected mainly in the Afrotropical region using two mitochondrial genes (COI and 16S rDNA) and two nuclear genes (CAD and 28S rDNA) to investigate the monophyly of 3 subgenera: *Avaritia* Fox, 1955, *Remmia* Glukhova, 1972 and *Synhelea* Kieffer, 1925, and the Milnei, Neavei and Similis species groups and (ii) delineating species boundaries using bPTP (Bayesian Poisson Tree Processes) and bGMYC (Bayesian General Mixed Yule Coalescent) methods. This work will revise the current internal systematic classification of *Culicoides* and help future work on the identification of *Culicoides* species in the Afrotropical region.

Materials and Methods

Culicoides collection and morphology

Culicoides specimens were collected in 23 sites located in 12 countries of the Afrotropical region (Fig. 1) and 2 sites in Lebanon (Palearctic region). *Culicoides* were collected through different field missions between 2009 and 2016 with OVI and CDC light

traps set at farms or near equids. Specimens were preserved in 70% ethanol, identified and sexed under a binocular microscope using the available identification keys for the region (Boorman, 1989; Glick, 1990; Cornet & Brunhes, 1994; Labuschagne, 2016). Based on the world systematic catalog of *Culicoides* species (Borkent, 2016) and Labuschagne's classification (Labuschagne, 2016), specimens belonging to the subgenera *Avaritia* Fox, 1955; *Remmia* Glukhova, 1972; *Synhelea* Kieffer, 1925; and the Milnei, Neavei and Similis groups were considered in this study. All specimens morphologically identified (or closely related) as species belonging to the above mentioned subgenus or groups were kept. For each specimen, the wings and genitalia were dissected prior to DNA extraction processing and slide-mounted to record the morphological features. All samples are kept as a reference at Cirad, UMR117 ASTRE, Montpellier, France and are available upon request to the corresponding authors.

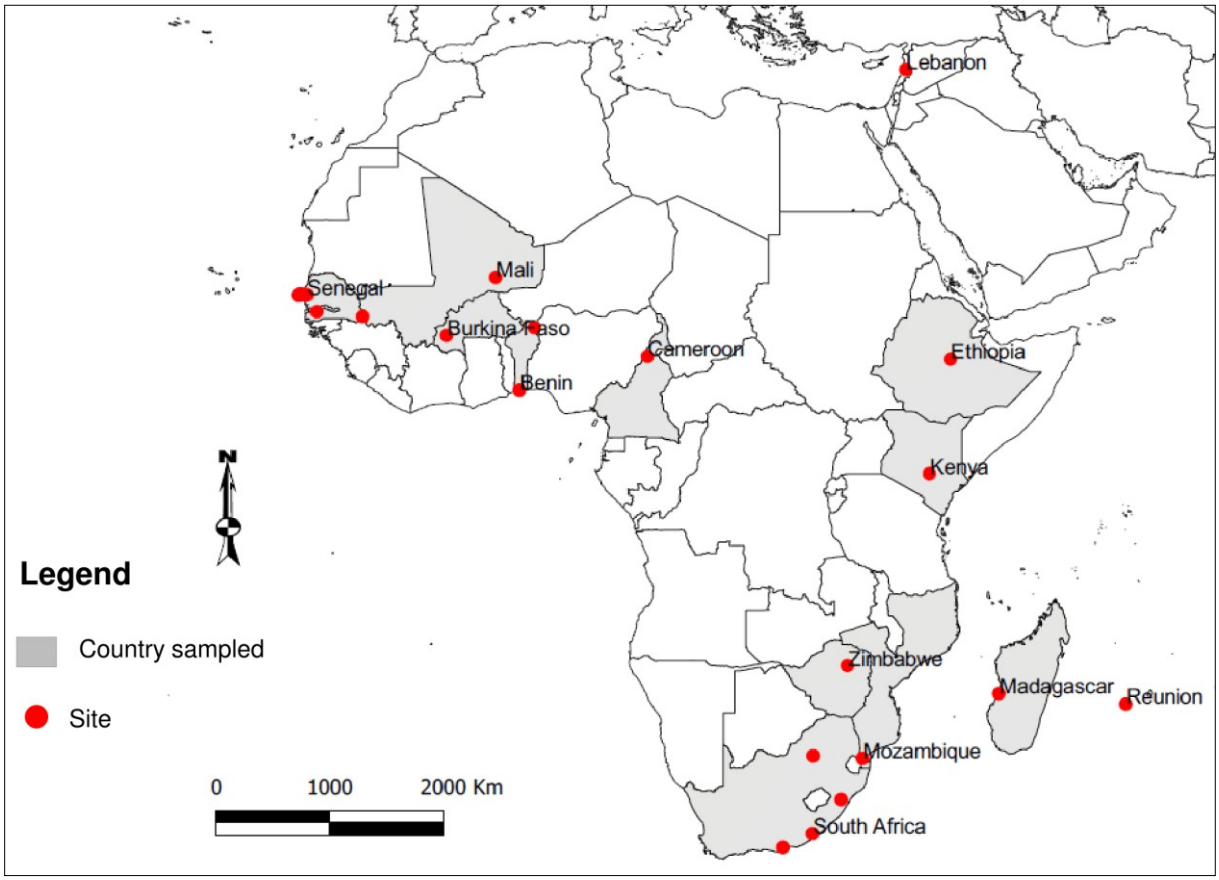

Fig. 1. Geographic map of the *Culicoides* sampling sites

DNA extraction, amplification and sequencing

Thorax of the *Culicoides* was individually homogenized in 50 µL of PBS 1X. After crushing using a piston pellet, genomic DNA was extracted using the *NucleoSpin® Tissue*

DNA Kit (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's instructions and maintained at –20°C until further use.

Four fragments from cytochrome oxidase subunit I (COI), 16S ribosomal DNA (16S rDNA), 28S ribosomal DNA (28S rDNA) and CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase) genes were amplified through Polymerase Chain Reaction (PCR) (Table 1). PCR amplification reactions were performed in a 25 µL total reaction volume containing 1X of Qiagen buffer, 1 mM of $MgCl^2$, 0.25 mM of each dNTP, 0.2 µM of each primer, 1.25 U of Qiagen Polymerase Taq and 0.4 or 0.7 ng/µL of genomic DNA depending the gene (COI, 16S rDNA and 28S rDNA genes or CAD gene).

Step-up PCR programs for COI, 16S rDNA and 28S rDNA included 1 step of 5 cycles before final step with 35 cycles. The PCR cycling conditions were as follows: an initial denaturation step at 94 °C for 5 min followed by 5 cycles of 94°C for 30 s, (45°C for COI, 42°C for 16S rDNA, 55°C for 28S rDNA) for 40 s, 72°C for 1 min, 35 cycles of 94 °C for 30 s, (51°C for COI, 55°C for 16S rDNA, 50°C for 28S rDNA) for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The touch-down amplification PCR conditions for CAD involved 2 steps of 4 of 94°C for 30 s, 51°C for 40 s, 72°C for 1 min and 6 cycles of 94°C for 30 s, 47°C for 40 s, 72°C for 1 min followed by 30 cycles of 94°C for 30 s, 42°C for 40 s, 72°C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products were separated on 1.5% agarose gels for quality control and the remaining 20 µL were sequenced using the same primers as used in PCR amplifications (https://www.genewiz.com). All sequences were deposited in GenBank (Table S1): COI (MF399674-MF399811); 16S rDNA (MF422796 - MF422942); 28S rDNA (MF422943 - MF423087) and CAD (MF399674-MF399811).

449 **Table 1**. Primers used for PCRs and sequencing in this study

450

Gene	Primer name	Sequence (5'–3')	Length of amplified fragment (bp)	References
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	710	Folmer et al. 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		
16S rDNA	16SF1	CACGTAAGAACTAAATAGTCGAAC	450	Ekrem et al. 2010
	16SR1	GACCGTGCAAAGGTAGCATAATC		
28S rDNA	28S_S3660	GAG AGT TMA ASA GTA CGT GAA AC	657	Dowton & Austin 1998
	28S_A335	TCG GAA GGA ACC AGC TAC TA		Whiting et al. 1997
CAD	TB1.F	TTGGCCGTAAGTTCGAGGAAG	674	This study
	TB1.R	AGTTCACGCAAACATCCAACG		
	787F	GGD GTN ACN ACN GCN TGY TTY GAR CC	905	Moulton and Wiegmann 2004
	1098R	TTN GGN AGY TGN CCN CCC AT		

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Sequence analysis

Amplified sequences were used as query in a BLAST search in the NCBI database to confirm that the amplified sequences were the target genes. The DNA sequences were edited in Geneious R6 (Biomatters, http://www.geneious.com/). Sequences of each gene were independently aligned using MACSE (Multiple Alignment of Coding SEquences accounting for frame shifts and stop codons) (Ranwez *et al.*, 2011) for COI and CAD genes. For 16S rDNA and 28S rDNA sequences, alignments were generated using Muscle (Edgar, 2004). For each alignment, segments that had too many variable positions or gaps were removed using Gblocks 0.91b (Castresana, 2000) to make alignments more appropriate for phylogenetic reconstruction. The reading frames and sequence statistics were checked under MEGAv6.0 (Tamura *et al.*, 2013). Test of substitution saturation (Xia *et al.*, 2003) was performed in DAMBE (Xia & Xie, 2001). Rapid detection of selective pressure on individual sites of codon alignments for CAD gene was performed using Datamonkey (Pond & Frost, 2005). Sites under positive or negative selection in this gene were inferred using the single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), mixed effects model of evolution (MEME) and Fast Unconstrained Bayesian AppRoximation (FUBAR) methods as implemented in Datamonkey server (http://www.datamonkey.org) (Pond & Frost, 2005; Murrell *et al.*, 2012). Positive selection for a site was considered to be statistically significant if the p-value was <0.1 for the SLAC, FEL and MEME methods or the posterior probability was <0.9 for the FUBAR method. Selected sites with a p-value < 0.05 were reported.

Cytochrome Oxidase Subunit 1 sequences from *C. bolitinos* population from Reunion Island (Desvars *et al.*, 2010) [accession numbers: HQ447061.1 and HQ447062.1], *C. fulvus* population from Australasia (Gopurenko *et al.*, 2015) [accession numbers: KT352267.1, KT352340.1, KT352547.1, KT352629.1 and KT352696.1], *C. miombo* population from Benin (Mathieu *et al.*, 2013) [accession numbers: KF417704.1 and KF417705.1] and *C. similis* population from India (Harrup *et al.*, 2016) [accession numbers: KT307841.1-KT307842.1]; and CAD sequences from *C. kwagga*, *C. loxodontis*, *C. gulbenkiani*, *C. tuttifrutti* and *C. bolitinos* populations from South Africa [accession numbers: KJ163032.1, KJ163034.1, KJ163025.1, KJ163044.1, KJ163009.1]; and *C. imicola* from France [accession number: KJ163028.1] (Bellis *et al.*, 2013) were used in this study.

Phylogenetic inferences

Phylogenetic trees were reconstructed for the four markers using Bayesian Inference (BI) and Maximum Likelihood (ML) under a substitution model found using jModelTest

(Darriba *et al.*, 2012). The BIC implemented within jModelTest was used to determine the most suitable evolutionary model(s). We used BI and ML to reconstruct phylogenetic relationships of all sequenced specimens using separately COI, 16S rDNA, 28S rDNA and CAD genes. For the concatenated alignment including COI, 16S rDNA and 28S rDNA, the best-fit partitioning scheme and partition-specific substitution model were tested in PartitionFinder v1.1 (Lanfear *et al.*, 2012) using the *greedy* algorithm, and the *mrbayes* or *raxml* set of models. The BI analyses were performed using MrBayes 3.2.3 (Ronquist *et al.*, 2012). Two simultaneous and independent runs consisting of sixteen Metropolis-coupled Markov chain Monte Carlo (MCMC) running 50 million generations were used, with a tree sampling every 1000 generations to calculate posterior probabilities (PP). In order to investigate the convergence of the runs, we investigated the split frequencies and Effective Sample Size (ESS) of all the parameters, and plotted the log-likelihood of the samples against the number of generations in Tracer 1.5 (http://BEAST.bio.ed.ac.uk/Tracer). A value of ESS> 2710 was found as a good indicator of convergence. The ML analyses were conducted with the best model selected using PhyML 3.0 (Guindon S. *et al.*, 2010) for each dataset and RAxML for the concatenate dataset (Stamatakis, 2014). We performed 1000 bootstrap replicates to investigate the level of support at each node.

Molecular species delimitation

We used the Bayesian Poisson Tree Processes (bPTP) on a molecular phylogenetic tree constructed from COI, 16S rDNA and 28S rDNA genes concatenated and the Bayesian General Mixed Yule Coalescent (bGMYC) on 100 ultrametric trees from each gene in order to delimit *Culicoides* species. The PTP method (Zhang *et al.*, 2013) infers molecular clades based on our inferred molecular phylogeny. The analyses were conducted on the web server for PTP (available at http://species.h-its.org/ptp/) using the Bayesian topology as advocated for this method (Zhang *et al.*, 2013). The bGMYC (Reid & Carstens, 2012) is a Bayesian implementation of the GMYC method. This method searches in an ultrametric gene tree the threshold at which branching patterns represent coalescent events or speciation events (Pons *et al.*, 2006). We conducted the bGMYC model using ultrametric gene trees inferred in the BEAST 1.8.0 (Drummond *et al.*, 2012) without outgroups under a strict clock model and a Speciation: Yule Process Tree Model. The runs consisted of 10 million generations sampled every 1000 cycles. Convergence was assessed by ESS values. A conservative burn-in of 10% was performed after checking the log-likelihood curves in Tracer 1.5. As recommended by Reid and Carstens (2012), 100 trees sampled at intervals from the posterior distribution of

trees using LogCombiner 1.8.0 (Drummond *et al.*, 2012) were used to perform the bGMYC analyses. Species delimitation analyses were conducted in R using the package 'bGMYC'. The analyses consisted for each of the 100 trees selected of 250,000 generations with a burnin of 25,000 and a thinning parameter of 100 as performed in Toussaint et al. (2015).

Results

Culicoides species

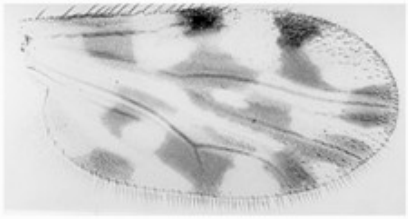
Based on morphological characteristics 47, 101 specimens were identified belonging to 58 *Culicoides* species. Of these, 153 specimens of 33 morphological units belonging to the *Avaritia*, *Remmia*, and *Synhelea* subgenera, Milnei, Neavei and Similis groups were selected. These 33 morphological units were distributed as follow: 16 belonging to *Avaritia*, 6 *Remmia* (Schultzei group: *C. enderleini*, *C. kingi*, *C. nevilli*, *C. oxystoma*, *C. subschultzei* and *C. schultzei*), 1 *C. tropicalis* Kieffer, 1913, type-species of *Synhelea*, 5 Milnei group (*C. austeni*, *C. isioloensis*, *C. milnei*, *C. moreli* and *C. zuluensis*), 2 Neavei group (*C. neavei* and *C. ovalis*) and 3 Similis Group (*C. exspectator*, *C. ravus* and *C. similis*). The sixteen species of the *Avaritia* were placed in the Dasyops group (*C. kanagai*, *C. sp*. #54 dark and pale forms), Grahamii group (*C. grahamii*), Gulbenkiani group (*C. gulbenkiani*), Imicola group (*C. bolitinos*, *C. imicola*, *C. kwagga*, *C. loxodontis*, *C. miombo*, *C. pseudopallidipennis*, *C. sp*. #22, and *C. tuttifrutti*) and Orientalis group (*C. trifasciellus* and *C. sp*. #20). *Culicoides kibatiensis* of the subgenus *Avaritia* was not grouped. Female wing pattern of these *Culicoides* species were described (Fig. 2).

Subgenus: *Avaritia* Fox, 1955

Diagnostics: Eyes contiguous. Sensilla coeloconica on each of flagellomeres: 1, 9-13 or 1, 10-13. The 3rd palpal segment is usually slender with a single sensory pit. Spermathecae: two ovoid well-developed with short necks, third rudimentary spermathecae and presence of a sclerotized ring. Short parameres and usually separate.

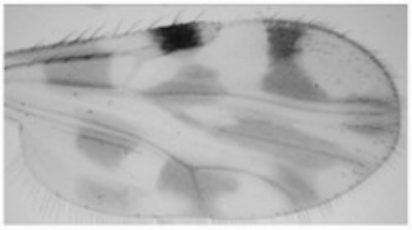
Imicola group

C. imicola Kieffer (♀):



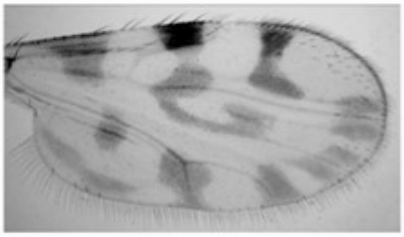
Proximal margin of pale spot in r3 cell is diamond shaped;
Vein M2 is dark at wing margin;
Pale spot above M2 vein is long and narrow.

C. bolitinos Meiswinkel (♀):



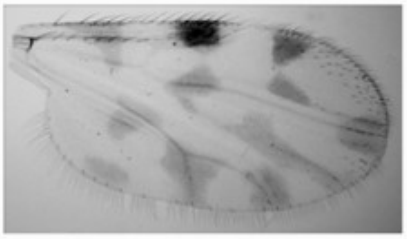
Proximal margin of 3rd post stigmatic pale spot in r3 cell is curved;
Spot over M2 vein at margin is narrowly dark.

C. kwagga (♀):



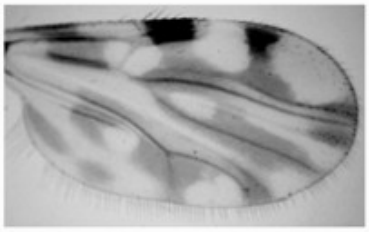
Proximal margin of pale spot in r3 cell is diamond shaped;
Dark spot at angle of anal cell is long.

C. loxodontis Meiswinkel (♀):



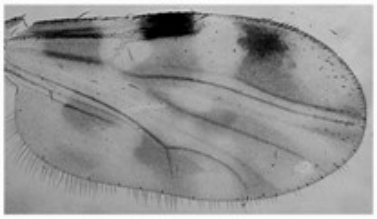
Wing pale with dark spots;
Proximal margin of pale spot in r3 cell is diamond shaped;
Area across M2 vein to wing margin is pale.

C. miombo Meiswinkel (♀):



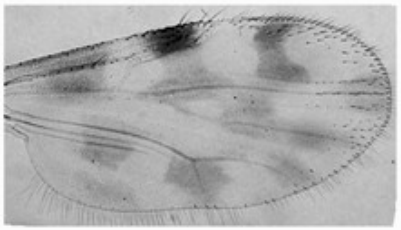
Wing dark with pale smudge;
Dark spot at angle of anal cell is long.

C. sp. # 22 (undescribed)



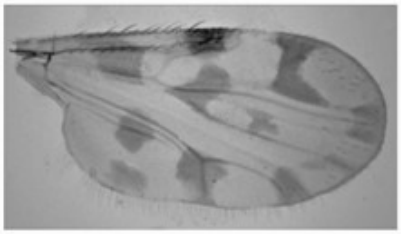
Similar to *C. bolitinos*;
Dark spot between 2nd costal spot and pale spot in r3 cell is trapezoid-shaped.

C. pseudopallidipennis Clastrier (♀)



Very similar to *C. imicola*;
Spot over M2 vein at margin is narrowly dark.

C. tuttifrutti Meiswinkel, Cornet and Dyce (♀)



Wing pattern very similar to that of *C. pseudopallidipennis* but spot over M2 vein is narrowly pale.

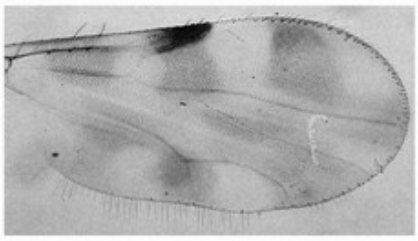
(Continued)

543

Subgenus: *Avaritia* Fox, 1955

Grahamii group

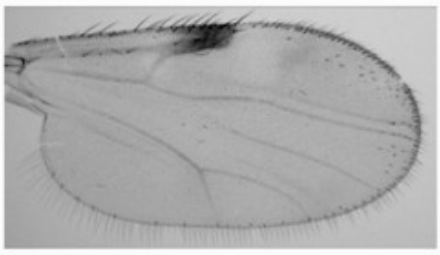
C. grahamii Goetghebuer (♀):



Dark wing with of pale spots;
1st costal spot across the r-m crossvein is round;
2nd costal spot is wider than the first costal spot;
Small pale spot in r3.

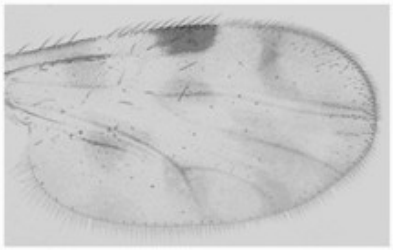
Dasyops group

C. kanagai Khamala and Kettle (♀):



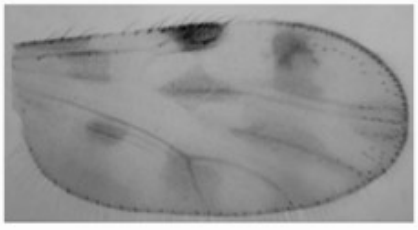
Very pale wing;
Dark area is on costal above and between r-m crossvein and 2nd radial cell.

C. sp. #54 pale form (undescribed)



Pale wing with faint pattern of dark spots;
Dark spot between pale spot in r3 cell and 2nd costal spot is thin.

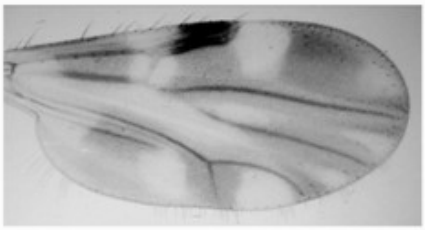
C. sp. #54 dark form (undescribed)



Similar to that of C. sp. # 54 pale form. Dark spot between spot r3 cell and 2nd costal spot is larger than in *C. sp.* #54 pale form.

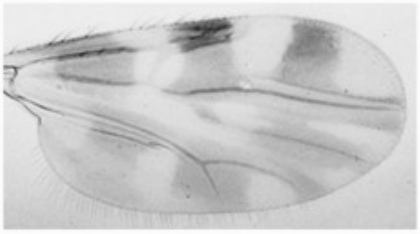
Orientalis group

C. trifasciellus Goetghebuer (♀):



1st costal spot is square and 2nd costal spot is round;
Dark spot between 2nd costal spot and small pale spot in r3 is large.

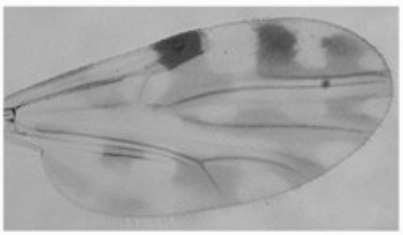
C. sp. # 20 (♀):



Very similar to *C.* trifasciellus;
Pale spot in r3 cell is wider than that of *C. trifasciellus*.

Gulbenkiani group

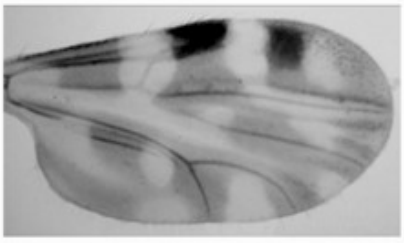
C. gulbenkiani Caeiro (♀):



Hourglass-shaped pale spot between 2nd costal spot and 3rd post stigmatic pale spot in r3 cell;
Tip of costal vein pale and intrudes into the 2nd costal spot.

Other Avaritia (No group)

C. kibatiensis Goetghebuer (♀):



Tip of 2nd radial cell pale and intrudes into the 2nd costal spot.
3rd post stigmatic pale spot in r3 cell does not touch the wing margin.

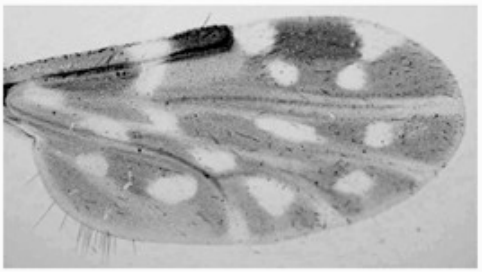
(Continued)

Subgenus: *Remmia* Glukhova, 1972

Diagnostics: Eyes narrowly to moderately separate. Sensilla coeloconica are normally on each of flagellomeres 1, 6-8 or 1, 3, 5-8 or 1, 5-8 and occasionally on 3 and 4. The 3rd palpal segment is moderately inflated with a single sensory pit. Distinct wing pattern with 3 to 4 pale spots in r3 cell, first two spots often connected to form an hourglass-shaped spot. Radial cells greatly reduced.

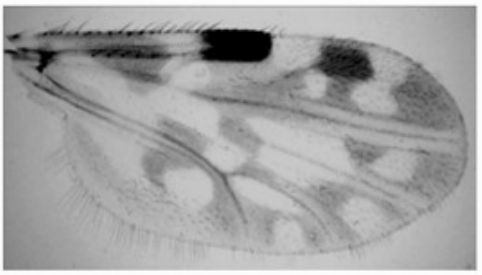
Schultzei group is the only one of this subgenus.

C. enderleini Cornet and Brunhes (♀):



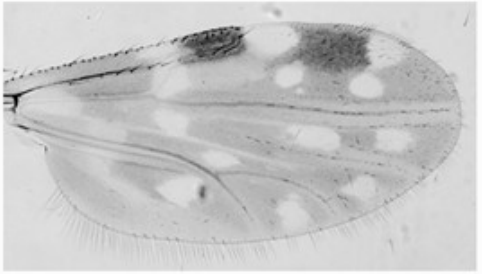
Pale spot in cua1 cell away from wing margin towards cubital fork;

C. kingi Austen (♀):



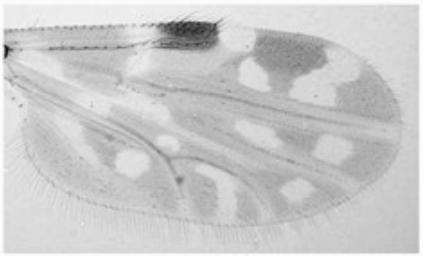
Two pale spots in cell cua1; m2 cell with a basal spot which runs over M2 vein and joins the basal spot in cell m1;
Tips of CuA1 and CuA2 veins are dark-bordered at wing margin.

C. nevilli Cornet and Brunhes (♀):



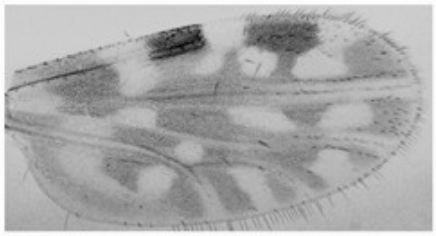
Pale spot in cua1 cell on wing margin;
Tips of CuA1 and CuA2 veins are dark-bordered at wing margin.

C. subschultzei Cornet and Brunhes (♀):



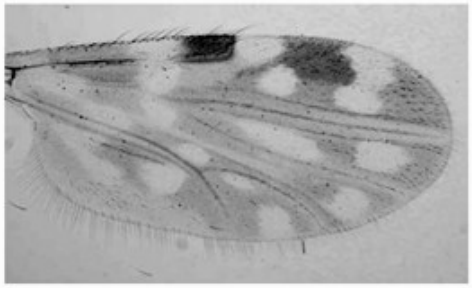
Pale spot in cua1cell long and narrow touch wing margin but not CuA1 vein.

C. oxystoma Kieffer (♀):



Wing pattern very similar to that of *C. subschultzei*;
Small pale spot under 2nd radial cell;
Tips of CuA1 and CuA2 veins are pale-bordered at wing margin.

C. schultzei Enderlein



Two pale spots in cua1cell: one is round and touches the wing margin and the other is long next to CuA1 vein.

(Continued)

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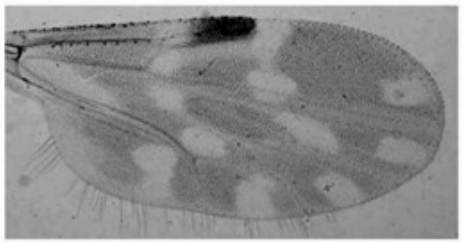
Tropicalis group (*Synhelea* subgenus Kieffer):

Diagnostics: Eyes separated. The 3rd palpal segment is usually slender with a single deep sensory pit. Sensilla coeloconica on each of flagellomeres varies within the group. Spermathecae: two ovoid well developed spermathecae with short necks, 3rd rudimentary spermathecae and presence of sclerotized ring.

C. tropicalis Kieffer (♀):

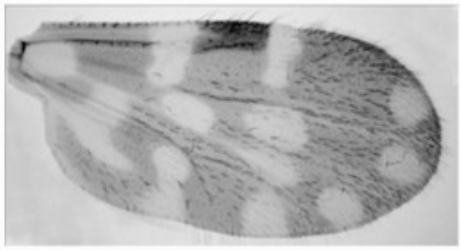


Pale spot below 2nd costal spot crosses vein M1.
1st costal spot does not cross r-m crossvein
.

Neavei group

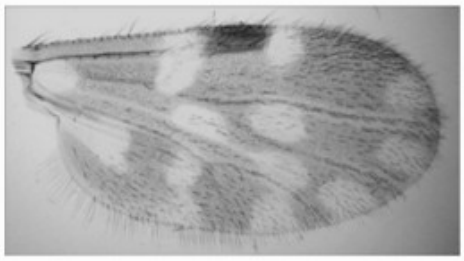
Diagnostics: Eyes separated moderately. No pale spot between 1st and 2nd costal spots. Spermathecae: 2 ovoid spermathecae with 3rd rudimentary spermathecae and sclerotized ring present. Sensilla coeloconica on each of flagellomeres: 1, 8-12 or 1, 9-12.

C. neavei Austen (♀):



Pale spots in r3, m1, m2, cua1 and anal cells touch wing margin.

C. ovalis Khamala & Kettle (♀):

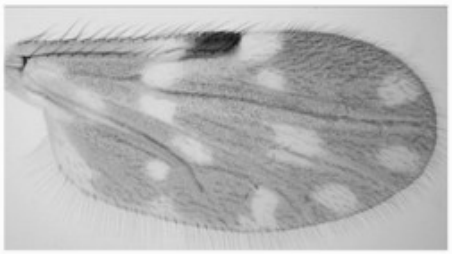


Similar to *C. neavei* but the 2nd costal spot is smaller and round;
Pale spots in basal half of m1 and m2 cells are more square-shaped.

Similis group

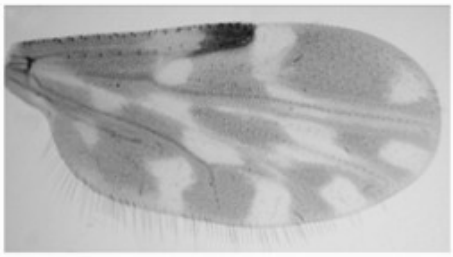
Diagnostics: Eyes very narrowly separated. The 3rd segment of the maxillary palpus inflated with single large, deep sensory pit. Sensilla coeloconica on each of flagellomeres: 1, 3, 5-8 or 1, 5-8 or 1, 8-12 or 1-8. Spermathecae: 2 ovoid spermathecae with 3rd rudimentary spermathecae and sclerotized ring present.

C. similis Carter, Ingram and Macfie (♀):



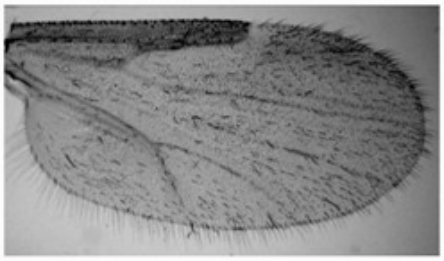
Small pale spots in r2, m1 and m2 cells;
Pale spot slightly below 2nd costal spot does not cross vein M1.

C. exspectator Clastrier (♀):



Wing with pale streaks situated just above and below middle of vein M2;
Pale spot below the 2nd costal spot touches and merges with vein M1 to form a pale streak.

C. ravus de Meillon (♀):



No pale markings on the wing;

.

(Continued)

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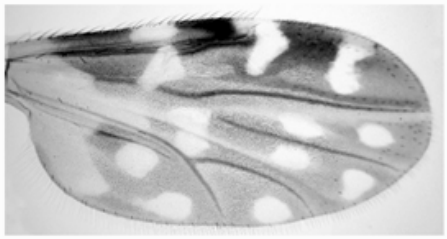
547

Milnei group:

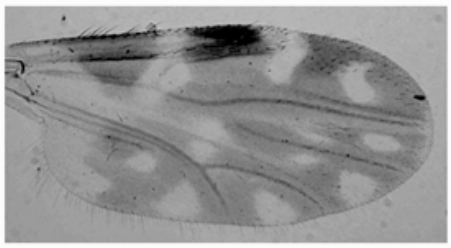
Diagnostics: Wing with prominent pattern of distinct pale spots; distal portion of 2nd radial cell is pale. Spermathecae: two ovoid well-developed with 3rd rudimentary spermathecae and sclerotized ring present at junction of ducts. Sensilla coeloconica on each of flagellomeres: 1, 9-13 or 3, 11-15. The 3rd segment of maxillary palpus is with more than 1 sensory pit.

C. milnei Austen (♀):



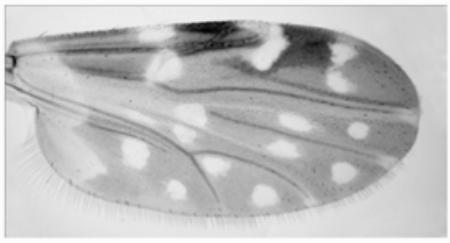
1st costal spot triangular and merges with pale spot above costal vein;
Pale spots in r3, m1 and m2 cells do not touch wing margin;
Single pale spot in cua1 cell on the wing margin.

C. austeni Carter (♀):



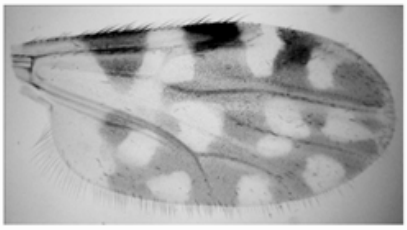
Very similar to that of *C. milnei*;
Pale spots on either side of the middle of the M2 vein are reduced.

C. moreli Clastrier (♀):



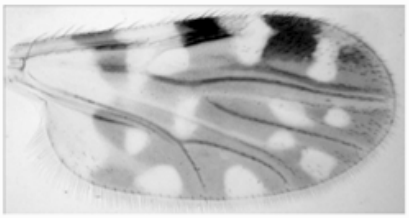
Two pale spots in cua1 cell: one near wing margin and the other at junction of veins CuA2 and CuA1 (cubital fork);
Tips of veins M1, M2, CuA1 are pale.

C. isioloensis Cornet, Nevill and Walker (♀):



Double spot straddling vein M2, midway between base and tip of vein and these are connected with streaks to spot in cell m;
Apex of wing pale between M1 and M2 veins;
Two equal pale spots in anal cell, one at wing margin other near vein;
Single pale spot in cua1 cell on the wing margin.

C. zuluensis de Meillon



Tips of CuA1, CuA2, M1 and M2 veins are dark;
1st costal spot large and square;
Single pale spot in cua1 cell on the wing margin.

Fig. 2. Female wing pattern of *Culicoides* species of *Avaritia* subgenus Fox, 1955; *Remmia* subgenus Glukhova, 1972; *Synhelea* subgenus Kieffer, 1925; and Milnei, Neavei and Similis groups included in our study. The wings were photographed using a x4 lens. Bars = 200 µm.

552 *DNA sequences*

553 DNA sequences generated in this study are deposited in GenBank (COI (MF399674-
554 MF399811); 16S rDNA (MF422796 - MF422942); 28S rDNA (MF422943 - MF423087) and
555 CAD (MF399674-MF399811). Of 153 samples considered in this study, we obtained 139
556 sequences for COI gene, 147 for 16S rDNA and 146 for 28S rDNA. Many samples failed
557 sequencing to the CAD gene (66 out of 153 samples amplified). The final concatenated
558 alignment of COI, 16S rDNA and 28S rDNA yielded 132 sequences of 1493 bp for 31
559 morphological units corresponding to all studied *Culicoides* species excluding *C. trifasciellus*
560 and *C. sp* #54 pale form. Information relative to sequence statistics and best-fit partitioning
561 scheme and partition-specific substitution model are provided in Tables 2 and 3. Rapid
562 detection of selective pressure on individual sites of 176 codons for CAD gene found 11
563 positively selected sites. These 11 sites were removed in order to make a better phylogenetic
564 tree using CAD gene. One hundred and sixty five negatively selected sites were also observed
565 for this gene. Saturation tests as a function of the genetic distance estimated under substitution
566 model JC69 showed low saturation of DNA sequence alignments.

567

568 **Table 2.** Sequence statistics of the four gene fragments

	COI	*16S* rDNA	*28S* rDNA	*CAD*
Lenght (pb)	567	259	667	495
Percentage C+G (%)	30.53	21.3	39.67	50
Number of variable sites	313	107	606	233
Number of parsimony informative sites	270	90	477	192

569

570 **Table 3.** Partition models and implemented parameters of the BI analysis

Partitioned dataset	Nucleotide model under BIC	Implemented model and related parameters
①*COI 1st*	GTR + I + G	nst = 6, rates = invgamma
②*COI 2nd*	GTR + I + G	nst = 6, rates = invgamma
③*COI 3rd*	GTR + I + G	nst = 6, rates = invgamma
④*16SrDNA*	GTR + I + G	nst = 6, rates = invgamma
⑤*28SrDNA*	HKY + I + G	nst = 2, rates = invgamma

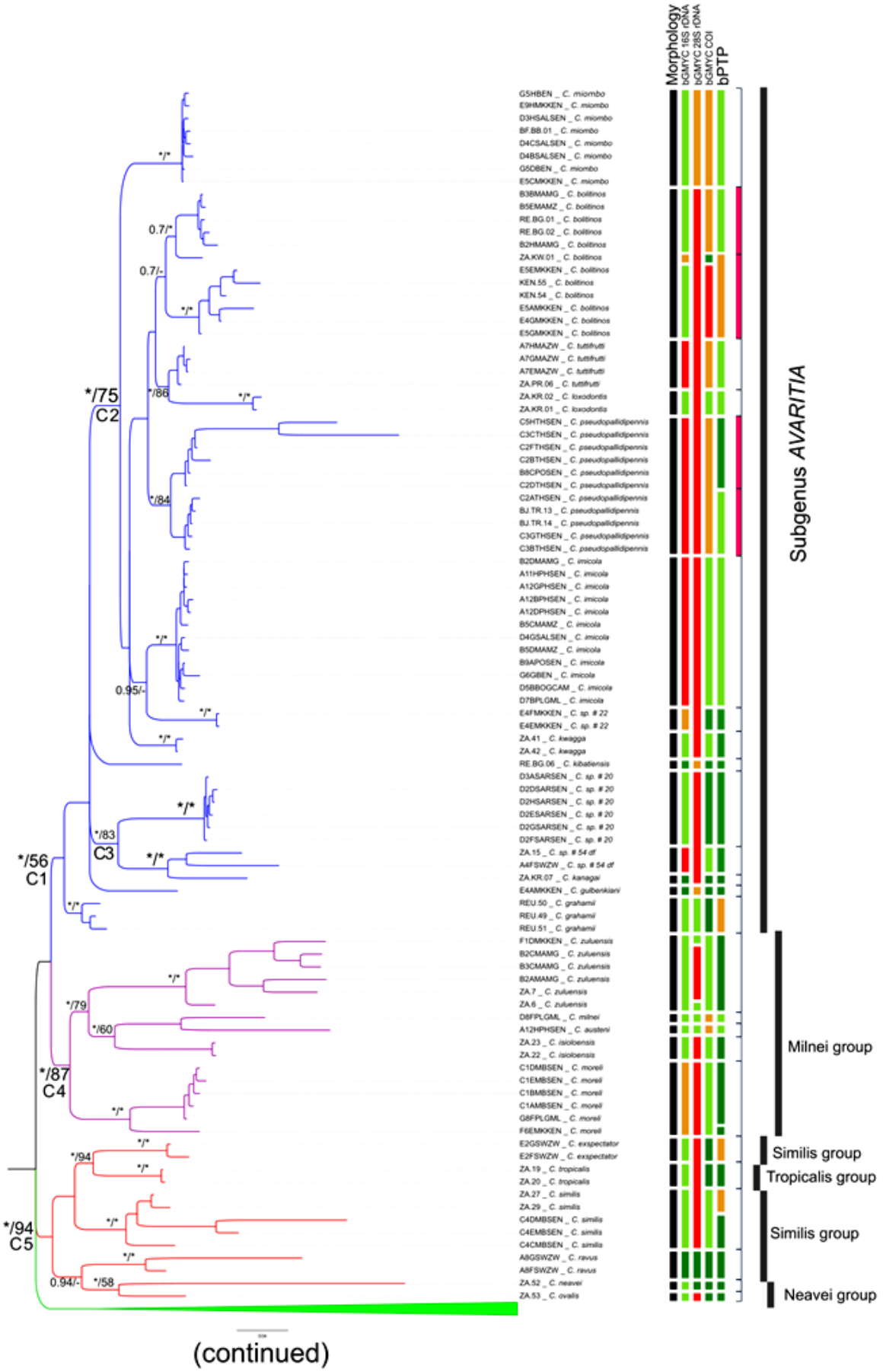
571 *Phylogenetic relationships*

572 Results from phylogenetic analyses conducted with COI, 16S rDNA and 28S rDNA
573 genes concatenated (Fig. 3) and CAD gene (Fig. 4) demonstrated clustering coherence. A
574 concatenated COI, 16S rDNA and 28S rDNA alignment of 132 specimens which included all
575 studied *Culicoides* species (Fig. 2) excluding *C. trifasciellus* and *C. sp* #54 pale form,
576 contained six clades: C1 to C6 (Fig. 3). CAD alignment of 72 specimens, including 6
577 sequences from GenBank: KJ163009.1, KJ163025.1, KJ163028.1, KJ163032.1, KJ163034.1
578 and KJ163044.1 (Bellis *et al.*, 2013), corresponding to 22 species [*C. bolitinos*, *C.*
579 *gulbenkiani*, *C. imicola*, *C. kanagai*, *C. kibatiensis*, *C. kwagga*, *C. loxodontis*, *C. miombo*, *C.*
580 *pseudopallidipennis* and *C. tuttifrutti* (*Avaritia*), *C. austeni*, *C. milnei*, *C. moreli* and *C.*
581 *zuluensis* (Milnei group), *C. enderleini*, *C. kingi*, *C. nevilli*, *C. oxystoma*, *C. schultzei* and *C.*
582 *subchultzei* (*Remmia*), *C. tropicalis* (*Synhelea*), and *C. similis* (Similis group)] contained 4
583 clades: C'1 to C'4 (Fig. 4). The clades C1 (Fig. 3) and C'1 (Fig. 4) contained all the
584 specimens affiliated to the subgenus *Avaritia*, which confirmed its monophyly. Regarding the
585 Fig. 3, *Culicoides grahamii* appeared at the basal position within the clade C1. The internal
586 subdivision of Clade 1 (*Avaritia*) is recovered as monophyletic with a strong support for the
587 two subclades (PP= 1/ BS= 75 for subclade C2, PP=1/BS=83 for subclade C3). The subclade
588 C2 contained all the studied species of the Imicola group (erected by Khamala & Kettle,
589 1971, and completed by Meiswinkel, 1995) as well as putative new species, *C. sp*. #22.
590 Species relationships within the Imicola Group (*C. bolitinos*, *C. imicola*, *C. kwagga*, *C.*
591 *loxodontis*, *C. miombo*, *C. pseudopallidipennis*, *C. sp*. #22, and *C. tuttifrutti*) were well
592 supported and resolved at the concatenated phylogenies constructed, but less so at the CAD
593 gene. *Culicoides gulbenkiani* sequence from GenBank and *C. kibatiensis* were nested among
594 the Imicola group in the CAD phylogeny (Fig. 4). The subclade C3 cluster includes two
595 groups with strong support: *C. sp*. # 20, affiliated to the Orientalis group, and specimens of
596 the Dasyops group, *C. sp*. # 54 dark form and *C. kanagai*. In the phylogenetic tree constructed
597 from the COI, relationship between *C. fulvus* population from Australasia and *C. sp.* #20 was
598 strong supported in Orientalis group (see Figure S1 for additional details). On the other hand,
599 *C. sp.* #20 was close to *C. trifasciellus* regarding phylogenetic tree constructed from 16S
600 rDNA gene (see Figure S2 for additional details). Phylogenetic relationships between *C.*
601 *kanagai*, *C. sp.* # 54 dark form and *C. sp*. #54 pale form were confirmed in phylogenetic trees
602 using 16S rDNA and 28S rDNA genes (see Figures S2 and S3 for additional details).
603 All the studied species of the Milnei group (*C. austeni*, *C. isioloensis*, *C. milnei*, *C. moreli* and
604 *C. zuluensis*) constituted a monophyletic clade with a strong support at the concatenated and

CAD phylogenies constructed (PP= 1/ BS= 87 for clade C4 in Fig. 3, PP=1/BS=100 for clade C'2 in Fig. 4). All phylogenies (concatenated and CAD phylogenies constructed) strongly supported the close relations of *C. tropicalis*, type species of subgenus *Synhelea* (Wirth et al. 1980), with the species of the Similis group. The clade C5 in Fig. 3 shows strong support (PP=1/BS=94) and contains *C.* (*Synhelea*) *tropicalis*, three species of the Similis group (*C. exspectator*, *C. ravus* and *C. similis*) and two species of the Neavei Group (*C. neavei* and *C. ovalis*). Regarding CAD phylogeny constructed (Fig. 4), clade C'3 (PP=1/BS=68) contains *C. tropicalis* and *C. similis*. All studied species of the subgenus *Remmia,* (Glukhova 1977) were well supported and congruently resolved in the concatenated and CAD phylogenies (PP= 1/ BS= 100 for clade C6 in Fig. 3, PP=1/BS=100 for clade C'4 in Fig. 4). The subgenus *Remmia* was recovered as monophyletic with strong support (1/100) and contained the 6 studied species (*C. enderleini*, *C. kingi*, *C. nevilli*, *C. oxystoma*, *C. subschultzei* and *C. schultzei*). The phylogenetic relationships between the different clades are not well supported and do not allow strong conclusions.

Molecular species delimitation

Using COI, 16S rDNA and 28S rDNA, we found that the number of putative species varied depending on the method and molecular markers used (Fig. 3). Based on bGMYC method with 16S rDNA and COI gene, results were similar unlike 28S rDNA that had a lower resolution. Analysis based on the bPTP method using concatenated phylogenetic tree yielded to very similar results to the clusters formed by morphological identification, but less so at the bGMYC method. Putative MOTUs (Molecular operation taxonomic units) were observed in *C. bolitinos*, *C. pseudopallidipennis*, and *C. oxystoma* (Fig. 3). The Cluster of *C. bolitinos* from Madagascar, Mozambique and Reunion (with posterior probability = 0.85) was separated from that *C. bolitinos* from South Africa and Kenya (PP = 0.65) (Fig. 3). We also observed a high level of divergence within *C. pseudopallidipennis* and *C. oxystoma* (Fig. 3). The bPTP analysis conducted on the concatenated phylogenetic tree showed two distinct clusters for *C. pseudopallidipennis* and also *C. oxystoma*. Some specimens of *C. pseudopallidipennis* from Senegal (PP = 0.98) were separated of *C. pseudopallidipennis* from Benin and some other specimens from Senegal (PP = 0.80). Molecular delineation of *C. oxystoma* based on the bPTP analysis using concatenated phylogenetic tree was very strong (Fig. 3). The cluster of *C. oxystoma* from Lebanon is separated from that of Senegal and Mali with posterior probabilities respectively equal to 1.0 and 0.98. However, all these putative MOTUs were not well supported by bGMYC conducted on individual genes.



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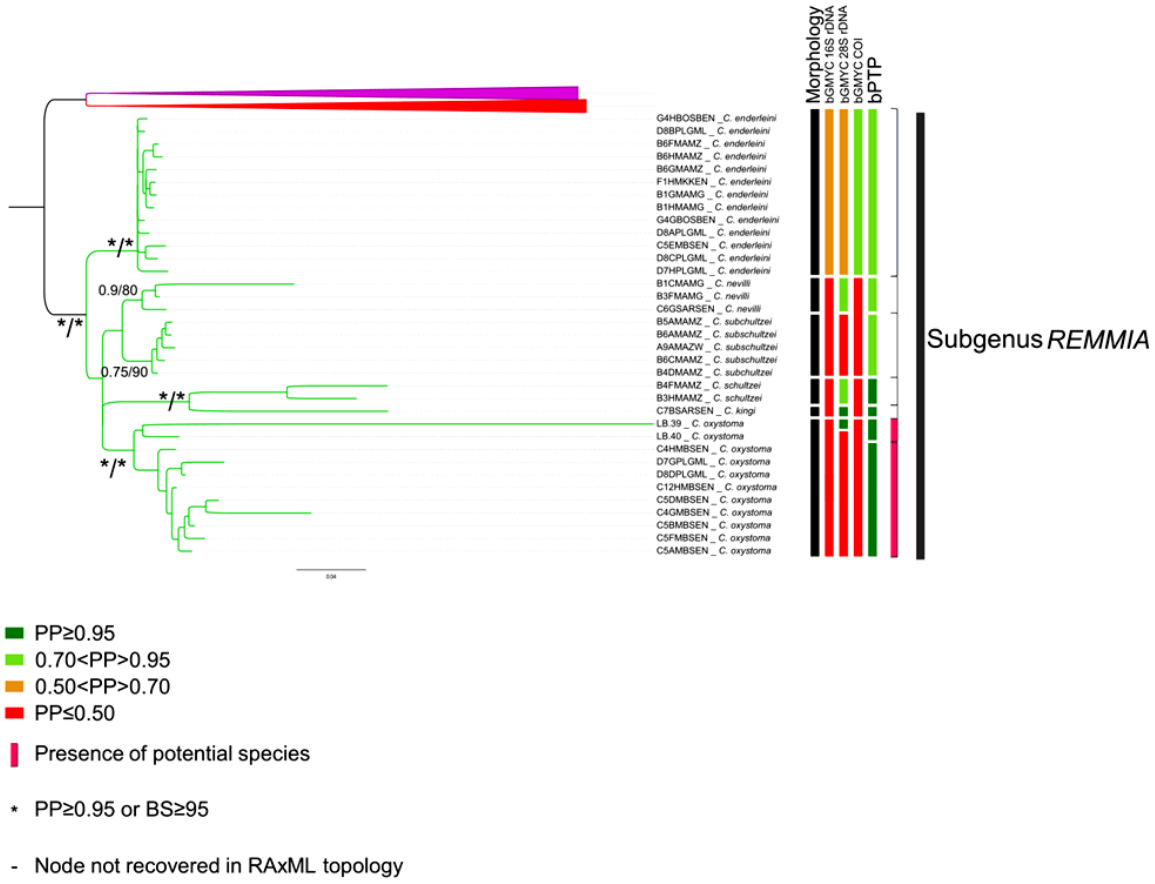

640

641 **Fig. 3.** *Culicoides* molecular phylogenetic relationships and species boundaries using COI, 16S rDNA and 28S
642 rDNA. Posterior probabilities and bootstrap values from the RAxML analysis are presented for the most
643 important nodes (asterisks indicate PPP 0.95 or BSP 95; –indicate that the node was not recovered in the
644 RAxML topology).

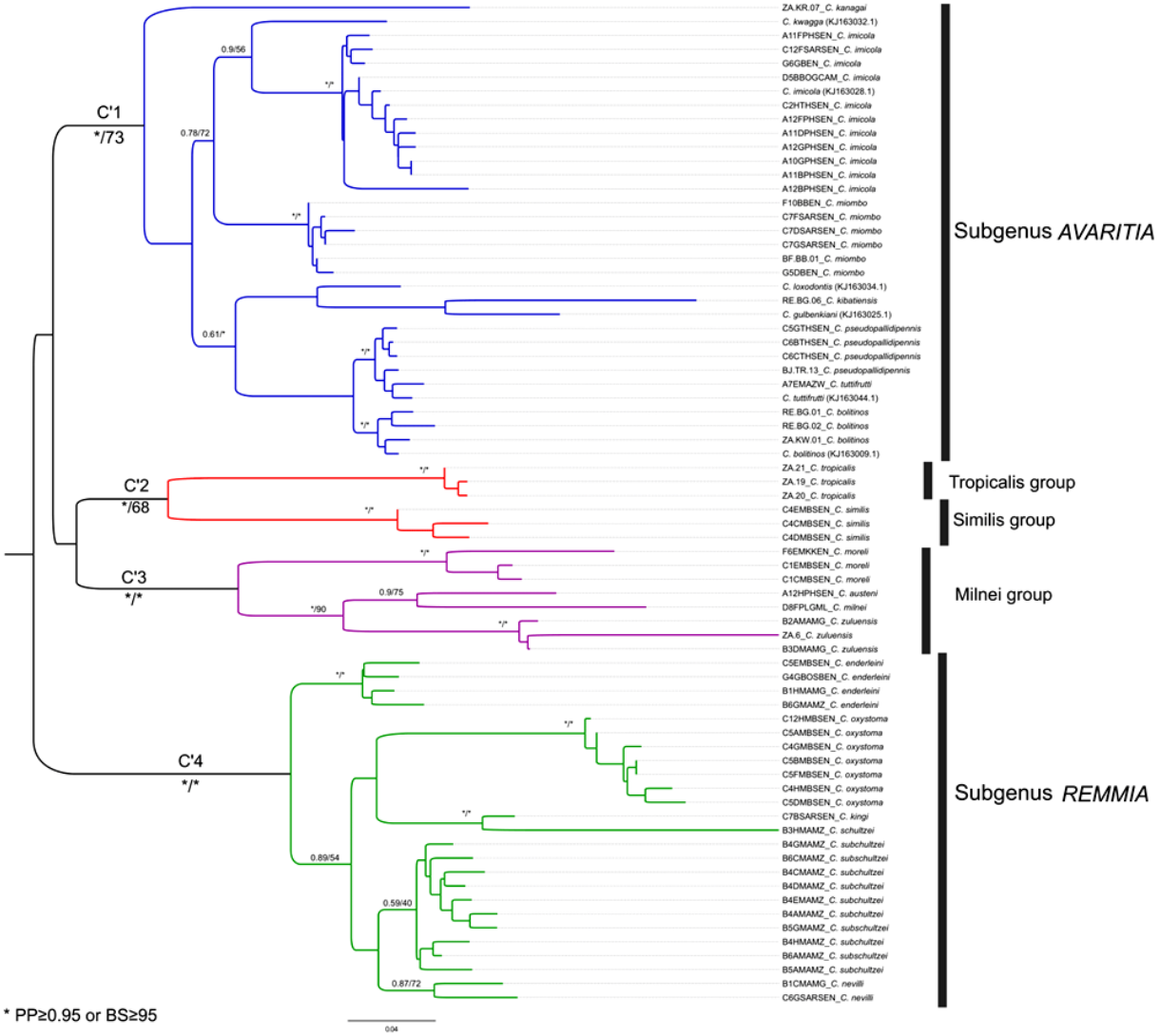

Fig. 4. *Culicoides* molecular phylogenetic relationships using CAD gene. Posterior probabilities and bootstrap values are presented for the most important nodes (asterisks indicate PPP 0.95 or BSP 95).

Discussion

The aim of this study was to demonstrate the monophyly of subgenera and groups of the genus *Culicoides*, previously described in the literature, with special interest for the Afrotropical region. In this study, we used integrative taxonomic approach incorporating four genes (COI, 16S rDNA, 28S rDNA and CAD) and morphological data to examine species boundaries as well phylogenetic relationships of *Avaritia*, *Remmia* and *Synhelea* subgenera, and Milnei, Neavei and Similis groups.

Because of missing sequences for the CAD gene, we performed separate phylogenetic analysis for CAD gene and concatenated COI, 16S rDNA and 28S rDNA genes. Missing data were observed for CAD gene due to amplification and sequencing difficulties despite several attempts with previous published primer sets (Moulton & Wiegmann, 2004; Bellis *et al.*, 2013; Gopurenko *et al.*, 2015). The relationships of *Culicoides* species of the first phylogenetic tree using CAD gene did not fit that generated by other genes or delimited by morphology. Moreover, rapid detection of selective pressure on individual sites of 176 codon for CAD gene found 11 positively selected sites and 165 negatively selected. Selective pressures may have the ability to generate phylogenetic signal that is different from ancestry (Massey *et al.*, 2008). Because of the amplification issue and the positive signal for selective pressure, unlike the other molecular markers studied, we do not consider CAD gene a good maker for phylogeny studies of Afrotropicale *Culicoides* species. Other primers sets from other arthropods groups could be use in future works (Sikes & Venables, 2013).

Phylogenetic tree using CAD gene without positively selected sites showed relationships of *Culicoides* subgenera or groups in line with the morphology. Indeed, positive selection has two possible modes of action on phylogenies: (1) increase in the positive selection rate causes long branch attraction, and (2) generates convergence or parallel evolution (homoplasy) through similar selective pressures (Philippe *et al.*, 2000; Massey *et al.*, 2008). Negative selection has also potential modes of action on phylogenies (Townsend, 2007; Massey *et al.*, 2008).

All species relationships within studied subgenera and groups were well supported and congruently resolved in the concatenated phylogenetic tree (COI, 16S rDNA and 28S rDNA), but less so for each gene individual. In concatenated and CAD phylogenetic trees, relationships between the different clades were not well supported and do not allow strong conclusions. In our study phylogenetic relationships and molecular delimitation species using bPTP on concatenated phylogenetic tree were in accord with that delimited by morphology, but less so with bGMYC based on COI, 16S rDNA and 28S rDNA genes, and showed

potential cryptic species within *C. bolitinos*, *C. pseudopallidipennis* and *C. oxystoma*. Cornet & Brunhes suggested that *Culicoides oxystoma* is a species complex (Cornet & Brunhes, 1994), and highest level of intra-specific divergence was observed in *C. oxystoma* based on COI sequences (Bakhoum *et al.*, 2013; Harrup *et al.*, 2016). In our opinion, further investigation of *C. oxystoma* specimens from the distribution area of this species (West Africa, Saharo-Arabian, Oriental and Australian regions) is necessary in order to delineate species within Oxystoma group.

The subgenus *Avaritia* was erected by Fox (1955). This definition was completed later, based on adult morphology (Blanton *et al.*, 1979; Wirth & Hubert, 1989). Several species belonging to *Avaritia* are of considerable veterinary importance as vectors of important arboviruses such as African horse sickness virus (AHSV), Bluetongue virus (BTV) and Epizootic hemorrhagic disease virus (EHDV) (Venter *et al.*, 1998; Meiswinkel *et al.*, 2004; Venter *et al.*, 2006b; Venter *et al.*, 2009). Molecular analyses using CAD gene and concatenated COI, 16S rDNA and 28S rDNA genes provided strong evidence that *Avaritia* subgenus includes species of Imicola group, *C. kibatiensis* (not grouped), *C. sp*. #20 (Orientalis group), species of Dasyops group (*C. kanagai* and *C. sp*. #54 dark form), *C. gulbenkiani* (Gulbenkiani group) and *C. grahamii* (Grahamii group). We demonstrate by the present study the monophyly of the subgenus *Avaritia* that is in agreement with previous studies (Perrin *et al.*, 2006; Augot *et al.*, 2017). The Imicola group including *Culicoides bolitinos*, *C. imicola*, *C. kwagga*, *C. loxodontis*, *C. miombo*, *C. pseudopallidipennis*, *C. tuttifrutti* and *C. sp.* #22 (a putative new species) is monophyletic. This monophyletic group is regarded as a natural species complex within the *Avaritia* subgenus (Meiswinkel, 1995; Bellis *et al.*, 2013). Based on adult characters, Meiswinkel (2004) separated the Imicola and Orientalis groups. Using morphological characters, *C. sp*. #20 from Senegal was closely related to species within the Orientalis Group. In the Afrotropical region, Orientalis group includes *C. brosseti*, *C. dubitatus* and *C. trifasciellus* (Kremer *et al.*, 1975; Meiswinkel, 2004). Relationships between *C. trifasciellus* and *C. sp.* #20 in phylogenetic trees constructed from 16S rDNA and 28S rDNA were strongly supported. Status of *C. sp*. #20 needs still to be clarified in the future studies. The Dasyops group, as suggested by Meiswinkel (1987) with a redescription of *C. kanagai* Khamala & Kettle 1971, is monophyletic and *C. kanagai* and *C. sp*. #54 dark form were well supported. Species within Dasyops group are typically Afrotropical. This group includes *C. alticola*; *C. kanagai*; *C. dasyops* and recently *C. sp*. # 54 Meiswinkel (new species, not described) (Nevill *et al.*, 2009; Labuschagne, 2016).

Relationships of *C. austeni*, *C. isioloensis*, *C. milnei*, *C. moreli* and *C. zuluensis* were well

supported. These species belong to the Milnei group, as defined by Cornet et al. (1974). Our molecular analyzes using CAD gene and concatenated COI, 16S rDNA and 28S rDNA genes indicated that Milnei group is monophyletic as previously reported by another study based on COI and 28S rDNA sequences (Augot *et al.*, 2017). Some species of the Milnei Group are of medical and veterinary interest (Labuschagne, 2016), such as *C. austeni* which is suspected in the transmission of *Mansonella perstans* to humans, and *C. milnei* of BTV to livestock (Labuschagne, 2016). According to Borkent's classification (Borkent, 2016), species of Milnei group are not in any subgenus whereas their morphological characters are similar to the subgenus *Hoffmania* Fox, 1948. Meiswinkel placed this group in the subgenus *Hoffmania* (Meiswinkel, 1996). Future investigations will take into account the morphological characteristics and molecular analyzes of the Milnei group and *Culicoides insignis* Lutz, type-species of the subgenus *Hoffmania* in order to place this group within the *Hoffmania* subgenus or to create a new subgenus for this monophyletic group.

Species relationships within the subgenus *Synhelea*, with *C. tropicalis* as type-species, were revised by Meiswinkel and Dyce (1989) based on morphological characters. They limited this subgenus to the Tropicalis group that include *C. camicasi*, C. *congolensis*, *C. dispar*, *C. dutoiti*, *C. moucheti*, *C. pellucidus*, *C. perettii*, *C. tauffliebi*, *C. tropicalis* and *C. vicinus* (Meiswinkel & Dyce, 1989). Regarding molecular analyzes using CAD gene, *C. tropicalis* is recovered in close relation with *C. similis* as sister taxons. Borkent's classification placed species from the Similis group in *Synhelea* subgenus (Borkent, 2016). Concatenated COI, 16S rDNA and 28S rDNA genes provide strong relationships between *C. tropicalis* and the Similis group (*C. exspectator* and *C. similis*). *Culicoides ravus* of Similis group is more closely related to *C. neavei* and *C. ovalis* of the Neavei group. These two species are recovered as sister taxon with *C. ravus*. *Culicoides tropicalis*, the Similis group (*C. exspectator*, *C. ravus* and *C. similis*) and the Neavei group (*C. neavei* and *C. ovalis*) are recovered as monophyletic with strong support (1.0/94). Based on our molecular analyzes Borkent's classification for the Similis group is maintained. And it is probable that the Neavei group, unplaced to subgenus (Borkent, 2016), belongs to subgenus *Synhelea*.

The subgenus *Remmia* is recovered as monophyletic with strong support (1.0/100) based on our molecular analyzes using CAD gene and concatenated COI, 16S rDNA and 28S rDNA genes. This subgenus, with *C. schultzei* as type-species, includes the Schultzei group with species of veterinary interest, such as *C. kingi* involved in the transmission of *Onchocerca gutturosa*, a widespread parasite of Sudanese cattle (El Sinnary & Hussein, 1980) or *C. oxystoma*, potential vector of Akabane virus in Japan (Kurogi *et al.*, 1987) and AHSV in

751 Senegal (Fall *et al.*, 2015; Bakhoum *et al.*, 2016). Molecular delineation using bPTP and
752 bGMYC methods for *C. oxystoma* from Lebanon and Senegal showed that *C. oxystoma* is a
753 complex of sibling species, as previously noted by several authors (Cornet & Brunhes, 1994;
754 Bakhoum *et al.*, 2013; Harrup *et al.*, 2016). In order to examine potential species within *C.*
755 *oxystoma*, we suggest to use Bayesian species delimitation implemented in Bayesian
756 Phylogenetics and Phylogeography (BPP) (Rannala & Yang, 2003; Yang & Rannala, 2010) in
757 the future investigations. This approach generates the posterior probabilities of species
758 assignments taking account of uncertainties due to unknown gene trees and the ancestral
759 coalescent process (Toussaint *et al.*, 2015).
760 We conclude that all species relationships within studied subgenera and groups were well
761 supported. However, we recorded a new species *C. sp.* #22 within the Imicola group which
762 was revealed monophyletic within the *Avaritia* subgenus. Milnei group was regarded as
763 monophyletic with strong support. Considered as monophyletic (Bakhoum *et al.*, 2013; Augot
764 *et al.*, 2017), monophyly of the Schultzei group (*Remmia* subgenus) was confirmed with *C.*
765 *oxystoma* as a potential complex of sibling species. In our study, all studied species of the
766 Similis group were placed in the subgenus *Synhelea* in accord with Borkent's classification
767 and should include *C. neavei* and *C. ovalis* of the Neavei group, which were closely related to
768 *C. ravus* of the Similis group.
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Supporting Information

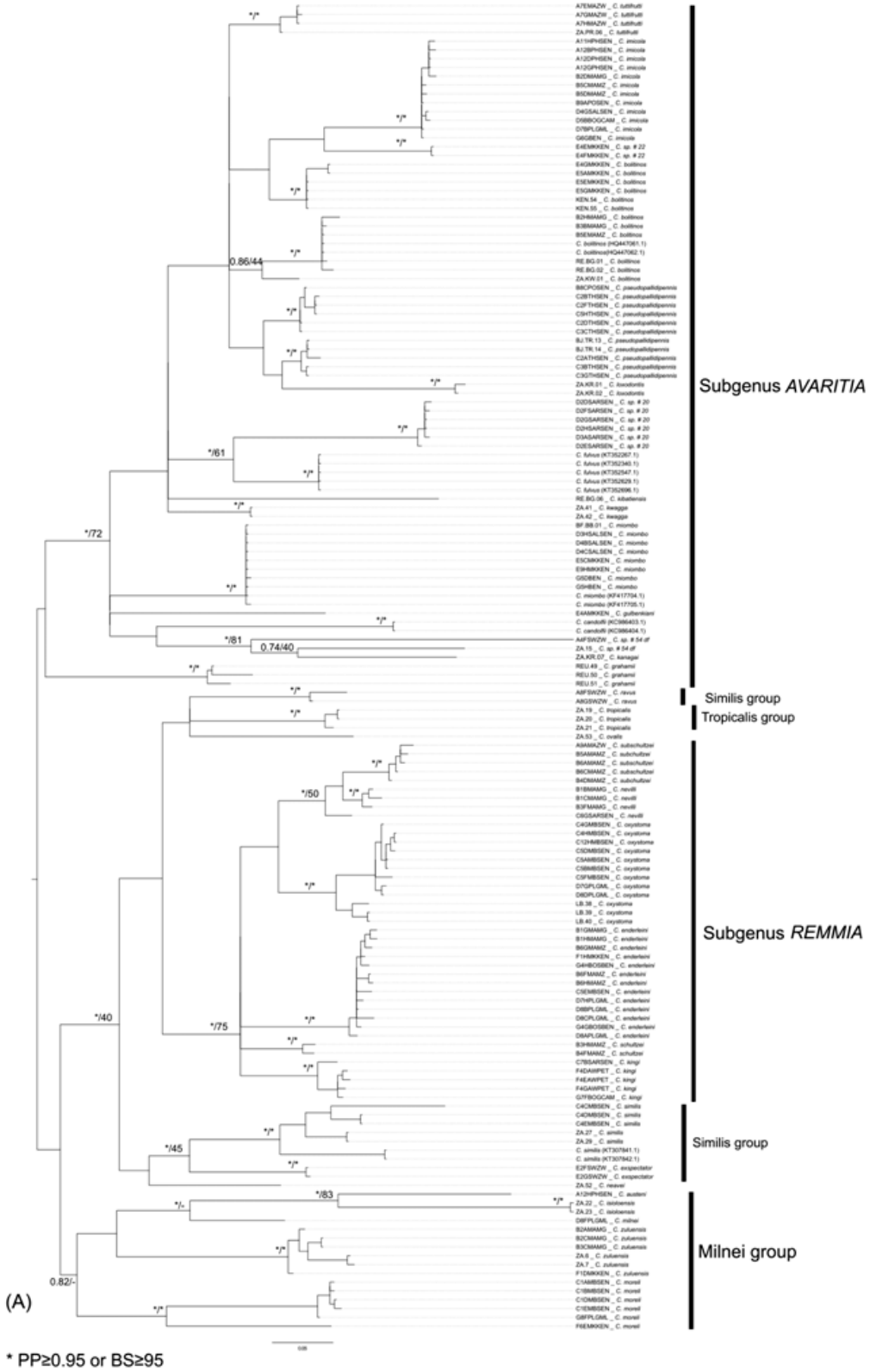

Figure S1. *Culicoides* molecular phylogenetic relationships using COI gene. Posterior probabilities and bootstrap values are presented for the most important nodes (asterisks indicate PPP 0.95 or BSP 95).

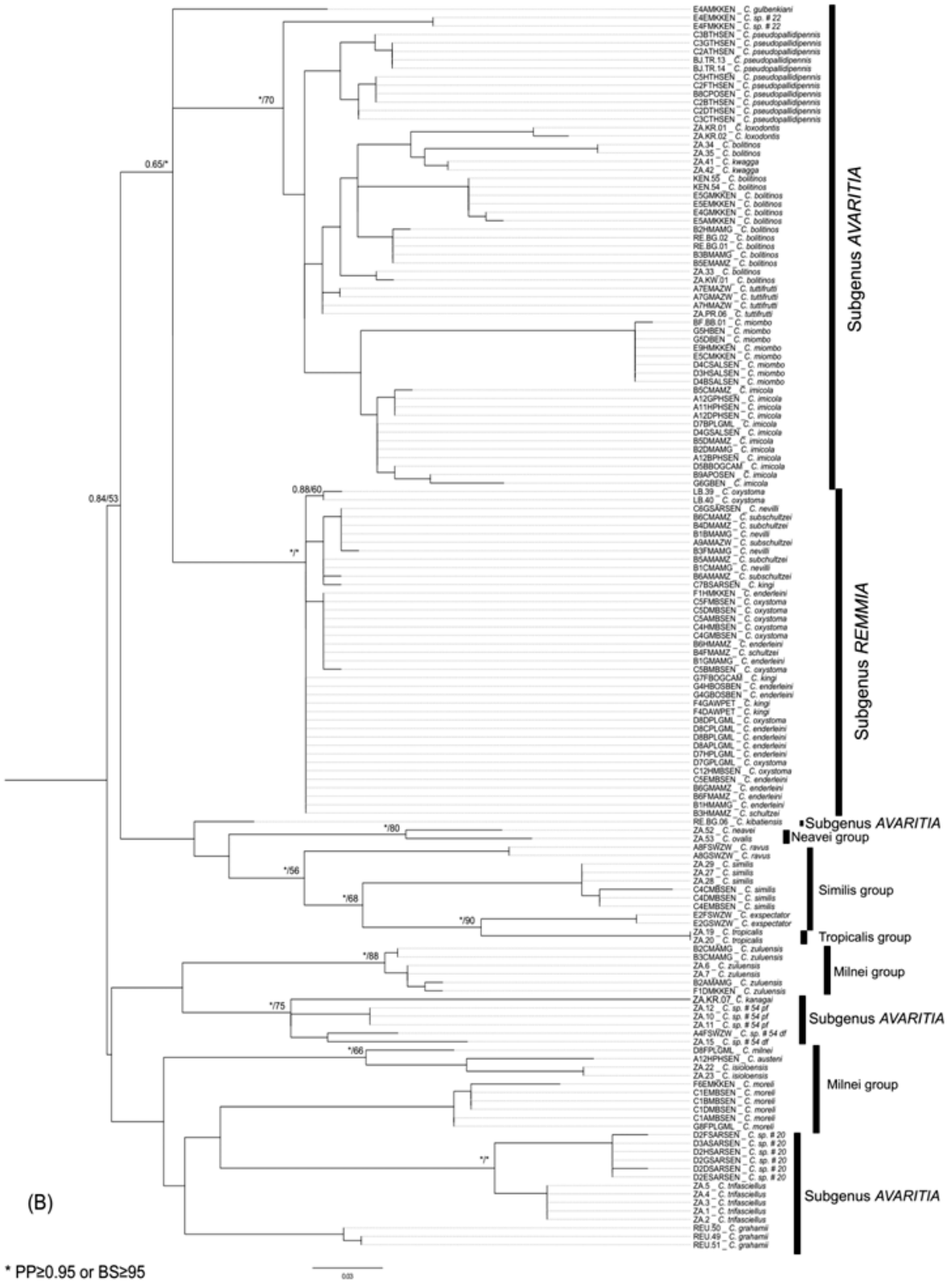

Figure S2. *Culicoides* molecular phylogenetic relationships using 16S rDNA gene. Posterior probabilities and bootstrap values are presented for the most important nodes (asterisks indicate PPP 0.95 or BSP 95).

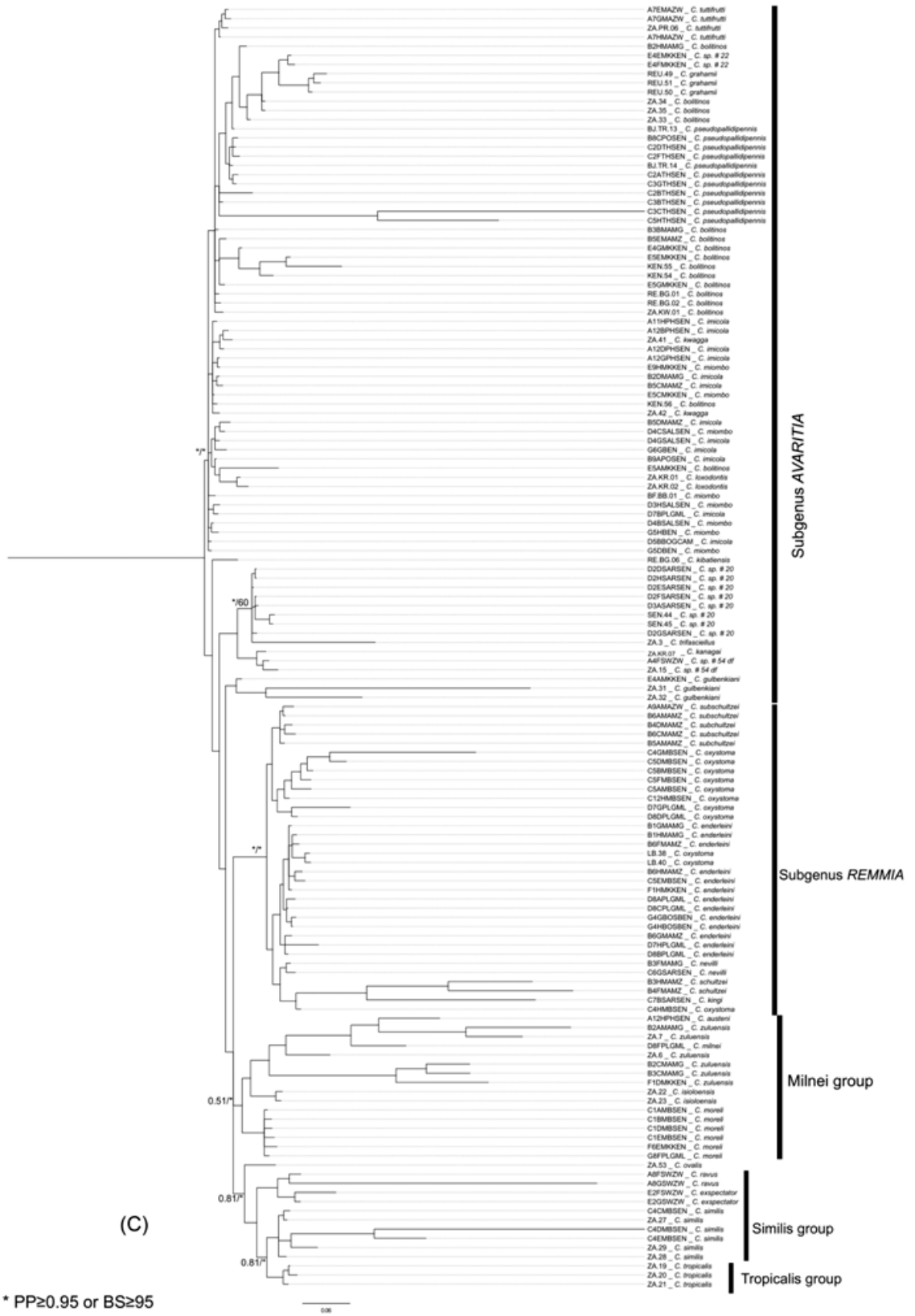

Figure S3. *Culicoides* molecular phylogenetic relationships using 28S rDNA gene. Posterior probabilities and bootstrap values are presented for the most important nodes (asterisks indicate PPP 0.95 or BSP 95).

Table S1 (1/6). *Culicoides* specimen details, including COI, 16S rDNA, 28S rDNA and CAD sequences (-indicate missing sequence), GenBank accession numbers, and sample location information

Culicoides species	Specimen ID	COI gene N° Accession	16S rDNA N° Accession	28S rDNA N° Accession	CAD gene N° Accession	Country	Locality
C. austeni	A12HPHSEN	submitted	submitted	submitted	submitted	Senegal	Parc de Hann
C. bolitinos	B2HMAMG	submitted	submitted	submitted	-	Madagascar	Masoandro
C. bolitinos	B3BMAMG	submitted	submitted	submitted	-	Madagascar	Masoandro
C. bolitinos	B5EMAMZ	submitted	submitted	submitted	-	Mozambique	Maputo
C. bolitinos	KEN.54	submitted	submitted	submitted	-	Kenya	Munukathi
C. bolitinos	RE.BG.01	submitted	submitted	submitted	submitted	France-Reunion Island	Le Tampon
C. bolitinos	RE.BG.02	submitted	submitted	submitted	submitted	France-Reunion Island	Le Tampon
C. bolitinos	ZA.33	-	submitted	submitted	-	South Africa	Port Elizabeth
C. bolitinos	ZA.34	-	submitted	submitted	-	South Africa	Port Elizabeth
C. bolitinos	ZA.35	-	submitted	submitted	-	South Africa	Port Elizabeth
C. bolitinos	ZA.KW.01	submitted	submitted	submitted	submitted	South Africa	Kwazulu-Natal
C. bolitinos	KEN.55	submitted	submitted	submitted	-	Kenya	Munukathi
C. bolitinos	KEN.56	-	-	submitted	-	Kenya	Munukathi
C. bolitinos	E4GMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. bolitinos	E5AMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. bolitinos	E5EMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. bolitinos	E5GMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. enderleini	B1GMAMG	submitted	submitted	submitted	-	Madagascar	Masoandro
C. enderleini	B1HMAMG	submitted	submitted	submitted	submitted	Madagascar	Masoandro
C. enderleini	B6FMAMZ	submitted	submitted	submitted	-	Mozambique	Maputo
C. enderleini	B6GMAMZ	submitted	submitted	submitted	submitted	Mozambique	Maputo
C. enderleini	B6HMAMZ	submitted	submitted	submitted	-	Mozambique	Maputo
C. enderleini	C5EMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. enderleini	D7HPLGML	submitted	submitted	submitted	-	Mali	Gao
C. enderleini	D8APLGML	submitted	submitted	submitted	-	Mali	Gao
C. enderleini	D8BPLGML	submitted	submitted	submitted	-	Mali	Gao
C. enderleini	D8CPLGML	submitted	submitted	submitted	-	Mali	Gao
C. enderleini	F1HMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. enderleini	G4GBOSBEN	submitted	submitted	submitted	submitted	Benin	Tori-Bossito
C. enderleini	G4HBOSBEN	submitted	submitted	submitted	-	Benin	Tori-Bossito
C. exspectator	E2FSWZW	submitted	submitted	submitted	-	Zimbabwe	Swimuwini
C. exspectator	E2GSWZW	submitted	submitted	submitted	-	Zimbabwe	Swimuwini
C. grahamii	REU.49	submitted	submitted	submitted	-	France-Reunion Island	Le Tampon
C. grahamii	REU.50	submitted	submitted	submitted	-	France-Reunion Island	Le Tampon

Table S1 (2/6). *Culicoides* specimen details, including COI, 16S rDNA, 28S rDNA and CAD sequences (-indicate missing sequence), GenBank accession numbers, and sample location information

Culicoides species	Specimen ID	COI gene N° Accession	16S rDNA N° Accession	28S rDNA N° Accession	CAD gene N° Accession	Country	Locality
C. grahamii	REU.51	submitted	submitted	submitted	-	France-Reunion Island	Le Tampon
C. gulbenkiani	E4AMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. gulbenkiani	ZA.31	-	-	submitted	-	South Africa	Komga
C. gulbenkiani	ZA.32	-	-	submitted	-	South Africa	Komga
C. imicola	A10GPHSEN	-	-	-	submitted	Senegal	Parc de Hann
C. imicola	A11BPHSEN	-	-	-	submitted	Senegal	Parc de Hann
C. imicola	A11DPHSEN	-	-	-	submitted	Senegal	Parc de Hann
C. imicola	A11FPHSEN	-	-	-	submitted	Senegal	Parc de Hann
C. imicola	A11HPHSEN	submitted	submitted	submitted	-	Senegal	Parc de Hann
C. imicola	A12BPHSEN	submitted	submitted	submitted	submitted	Senegal	Parc de Hann
C. imicola	A12DPHSEN	submitted	submitted	submitted	-	Senegal	Parc de Hann
C. imicola	A12FPHSEN	-	-	-	submitted	Senegal	Parc de Hann
C. imicola	A12GPHSEN	submitted	submitted	submitted	submitted	Senegal	Parc de Hann
C. imicola	B2DMAMG	submitted	submitted	submitted	-	Madagascar	Masoandro
C. imicola	B5CMAMZ	submitted	submitted	submitted	-	Mozambique	Maputo
C. imicola	B5DMAMZ	submitted	submitted	submitted	-	Mozambique	Maputo
C. imicola	B9APOSEN	submitted	submitted	submitted	-	Senegal	Pout
C. imicola	C12FSARSEN	-	-	-	submitted	Senegal	Saraya
C. imicola	C2HTHSEN	-	-	-	submitted	Senegal	Thies
C. imicola	D4GSALSEN	submitted	submitted	submitted	-	Senegal	Salikegne
C. imicola	D5BBOGCAM	submitted	submitted	submitted	submitted	Cameroon	Lanavet
C. imicola	D7BPLGML	submitted	submitted	submitted	-	Mali	Gao
C. imicola	G6GBEN	submitted	submitted	submitted	submitted	Benin	Ouidah
C. isioloensis	ZA.22	submitted	submitted	submitted	-	South Africa	Port Elizabeth
C. isioloensis	ZA.23	submitted	submitted	submitted	-	South Africa	Port Elizabeth
C. kanagai	ZA.KR.07	submitted	submitted	submitted	submitted	South Africa	Kruger
C. kibatiensis	RE.BG.06	submitted	submitted	submitted	submitted	France-Reunion Island	Le Tampon
C. kingi	C7BSARSEN	submitted	submitted	submitted	submitted	Senegal	Saraya
C. kingi	F4DAWPET	submitted	submitted	-	-	Ethiopia	Awash Park
C. kingi	F4EAWPET	submitted	-	-	-	Ethiopia	Awash Park
C. kingi	F4GAWPET	submitted	submitted	-	-	Ethiopia	Awash Park
C. kingi	G7FBOGCAM	submitted	submitted	-	-	Cameroon	Lanavet
C. kwagga	ZA.41	submitted	submitted	submitted	-	South Africa	Pretoria
C. kwagga	ZA.42	submitted	submitted	submitted	-	South Africa	Pretoria

Table S1 (3/6). *Culicoides* specimen details, including COI, 16S rDNA, 28S rDNA and CAD sequences (-indicate missing sequence), GenBank accession numbers, and sample location information

Culicoides species	Specimen ID	COI gene N° Accession	16S rDNA N° Accession	28S rDNA N° Accession	CAD gene N° Accession	Country	Locality
C. loxodontis	ZA.KR.01	submitted	submitted	submitted	-	South Africa	Kruger
C. loxodontis	ZA.KR.02	submitted	submitted	submitted	-	South Africa	Kruger
C. milnei	D8FPLGML	submitted	submitted	submitted	submitted	Mali	Gao
C. miombo	BF.BB.01	submitted	submitted	submitted	submitted	Burkina Faso	Bobo-Dioulasso
C. miombo	C7DSARSEN	-	-	-	submitted	Senegal	Saraya
C. miombo	C7FSARSEN	-	-	-	submitted	Senegal	Saraya
C. miombo	C7GSARSEN	-	-	-	submitted	Senegal	Saraya
C. miombo	D3HSALSEN	submitted	submitted	submitted	-	Senegal	Salikegne
C. miombo	D4BSALSEN	submitted	submitted	submitted	-	Senegal	Salikegne
C. miombo	D4CSALSEN	submitted	submitted	submitted	-	Senegal	Salikegne
C. miombo	E5CMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. miombo	E9HMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. miombo	F10BBEN	-	-	-	submitted	Benin	Ouidah
C. miombo	G5DBEN	submitted	submitted	submitted	submitted	Benin	Ouidah
C. miombo	G5HBEN	submitted	submitted	submitted	-	Benin	Ouidah
C. moreli	C1AMBSEN	submitted	submitted	submitted	-	Senegal	Mbao
C. moreli	C1BMBSEN	submitted	submitted	submitted	-	Senegal	Mbao
C. moreli	C1CMBSEN	-	-	-	submitted	Senegal	Mbao
C. moreli	C1DMBSEN	submitted	submitted	submitted	-	Senegal	Mbao
C. moreli	C1EMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. moreli	F6EMKKEN	submitted	submitted	submitted	submitted	Kenya	Munukathi
C. moreli	G8FPLGML	submitted	submitted	submitted	-	Mali	Gao
C. neavi	ZA.52	submitted	submitted	submitted	-	South Africa	Pretoria
C. nevilli	B1BMAMG	submitted	submitted	-	-	Madagascar	Masoandro
C. nevilli	B1CMAMG	submitted	submitted	submitted	submitted	Madagascar	Masoandro
C. nevilli	B3FMAMG	submitted	submitted	submitted	-	Madagascar	Masoandro
C. nevilli	C6GSARSEN	submitted	submitted	submitted	submitted	Senegal	Saraya
C. ovalis	ZA.53	submitted	submitted	submitted	-	South Africa	Nelspruit
C. oxystoma	C12HMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. oxystoma	C4GMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. oxystoma	C4HMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. oxystoma	C5AMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. oxystoma	C5BMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao

Table S1 (4/6). *Culicoides* specimen details, including COI, 16S rDNA, 28S rDNA and CAD sequences (-indicate missing sequence), GenBank accession numbers, and sample location information

Culicoides species	Specimen ID	COI gene N° Accession	16S rDNA N° Accession	28S rDNA N° Accession	CAD gene N° Accession	Country	Locality
C. oxystoma	C5DMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. oxystoma	C5FMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. oxystoma	D7GPLGML	submitted	submitted	submitted	-	Mali	Gao
C. oxystoma	D8DPLGML	submitted	submitted	submitted	-	Mali	Gao
C. oxystoma	LB.38	submitted	-	submitted	-	Lebanon	
C. oxystoma	LB.39	submitted	submitted	submitted	-	Lebanon	
C. oxystoma	LB.40	submitted	submitted	submitted	-	Lebanon	
C. pseudopallidipennis	B8CPOSEN	submitted	submitted	submitted	-	Senegal	Pout
C. pseudopallidipennis	BJ.TR.13	submitted	submitted	submitted	submitted	Benin	Tori-Bossito
C. pseudopallidipennis	BJ.TR.14	submitted	submitted	submitted	-	Benin	Tori-Bossito
C. pseudopallidipennis	C2ATHSEN	submitted	submitted	submitted	-	Senegal	Thies
C. pseudopallidipennis	C2BTHSEN	submitted	submitted	submitted	-	Senegal	Thies
C. pseudopallidipennis	C2DTHSEN	submitted	submitted	submitted	-	Senegal	Thies
C. pseudopallidipennis	C2FTHSEN	submitted	submitted	submitted	-	Senegal	Thies
C. pseudopallidipennis	C3BTHSEN	submitted	submitted	submitted	-	Senegal	Thies
C. pseudopallidipennis	C3CTHSEN	submitted	submitted	submitted	-	Senegal	Thies
C. pseudopallidipennis	C3GTHSEN	submitted	submitted	submitted	-	Senegal	Thies
C. pseudopallidipennis	C5GTHSEN	-	-	-	submitted	Senegal	Thies
C. pseudopallidipennis	C5HTHSEN	submitted	submitted	submitted	-	Senegal	Thies
C. pseudopallidipennis	C6BTHSEN	-	-	-	submitted	Senegal	Thies
C. pseudopallidipennis	C6CTHSEN	-	-	-	submitted	Senegal	Thies
C. ravus	A8FSWZW	submitted	submitted	submitted	-	Zimbabwe	Malipati
C. ravus	A8GSWZW	submitted	submitted	submitted	-	Zimbabwe	Malipati
C. schultzei	B3HMAMZ	submitted	submitted	submitted	submitted	Mozambique	Maputo
C. schultzei	B4FMAMZ	submitted	submitted	submitted	-	Mozambique	Maputo
C. similis	C4CMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. similis	C4DMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. similis	C4EMBSEN	submitted	submitted	submitted	submitted	Senegal	Mbao
C. similis	ZA.27	submitted	submitted	submitted	-	South Africa	Port Elizabeth
C. similis	ZA.28	-	submitted	submitted	-	South Africa	Port Elizabeth
C. similis	ZA.29	submitted	submitted	submitted	-	South Africa	Port Elizabeth
C. sp. # 20	D2DSARSEN	submitted	submitted	submitted	-	Senegal	Saraya
C. sp. # 20	D2ESARSEN	submitted	submitted	submitted	-	Senegal	Saraya

Table S1 (5/6). *Culicoides* specimen details, including COI, 16S rDNA, 28S rDNA and CAD sequences (-indicate missing sequence), GenBank accession numbers, and sample location information

Culicoides species	Specimen ID	COI gene N° Accession	16S rDNA N° Accession	28S rDNA N° Accession	CAD gene N° Accession	Country	Locality
C. sp. # 20	D2FSARSEN	submitted	submitted	submitted	-	Senegal	Saraya
C. sp. # 20	D2GSARSEN	submitted	submitted	submitted	-	Senegal	Saraya
C. sp. # 20	D2HSARSEN	submitted	submitted	submitted	-	Senegal	Saraya
C. sp. # 20	D3ASARSEN	submitted	submitted	submitted	-	Senegal	Saraya
C. sp. # 20	SEN.43	-	-	submitted	-	Senegal	Saraya
C. sp. # 20	SEN.44	-	-	submitted	-	Senegal	Saraya
C. sp. # 20	SEN.45	-	-	submitted	-	Senegal	Saraya
C. sp. # 20	SEN.47	-	-	submitted	-	Senegal	Saraya
C. sp. # 22	E4EMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. sp. # 22	E4FMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. sp. # 54 df	ZA.15	submitted	submitted	submitted	-	South Africa	Port Elizabeth
C. sp. # 54 df	A4FSWZW	submitted	submitted	submitted	-	Zimbabwe	Swimuwini
C. sp. # 54 pf	ZA.10	-	submitted	-	-	South Africa	Port Elizabeth
C. sp. # 54 pf	ZA.11	-	submitted	-	-	South Africa	Port Elizabeth
C. sp. # 54 pf	ZA.12	-	submitted	-	-	South Africa	Port Elizabeth
C. subchultzei	B4AMAMZ	-	-	-	submitted	Mozambique	Maputo
C. subchultzei	B4CMAMZ	-	-	-	submitted	Mozambique	Maputo
C. subchultzei	B4DMAMZ	submitted	submitted	submitted	submitted	Mozambique	Maputo
C. subchultzei	B4EMAMZ	-	-	-	submitted	Mozambique	Maputo
C. subchultzei	B4GMAMZ	-	-	-	submitted	Mozambique	Maputo
C. subchultzei	B4HMAMZ	-	-	-	submitted	Mozambique	Maputo
C. subchultzei	B5AMAMZ	submitted	submitted	submitted	submitted	Mozambique	Maputo
C. subschultzei	A9AMAZW	submitted	submitted	submitted	-	Zimbabwe	Malipati
C. subschultzei	B5GMAMZ	-	-	-	submitted	Mozambique	Maputo
C. subschultzei	B6AMAMZ	submitted	submitted	submitted	submitted	Mozambique	Maputo
C. subschultzei	B6CMAMZ	submitted	submitted	submitted	submitted	Mozambique	Maputo
C. trifasciellus	ZA.1	-	submitted	-	-	South Africa	Komga
C. trifasciellus	ZA.2	-	submitted	-	-	South Africa	Komga
C. trifasciellus	ZA.3	-	submitted	submitted	-	South Africa	Komga
C. trifasciellus	ZA.4	-	submitted	-	-	South Africa	Komga
C. trifasciellus	ZA.5	-	submitted	-	-	South Africa	Komga
C. tropicalis	ZA.19	submitted	submitted	submitted	submitted	South Africa	Port Elizabeth
C. tropicalis	ZA.20	submitted	submitted	submitted	submitted	South Africa	Port Elizabeth
C. tropicalis	ZA.21	submitted	-	submitted	submitted	South Africa	Port Elizabeth

Table S1 (6/6). *Culicoides* specimen details, including COI, 16S rDNA, 28S rDNA and CAD sequences (-indicate missing sequence), GenBank accession numbers, and sample location information

Culicoides species	Specimen ID	COI gene N° Accession	16S rDNA N° Accession	28S rDNA N° Accession	CAD gene N° Accession	Country	Locality
C. tuttifrutti	A7EMAZW	submitted	submitted	submitted	submitted	Zimbabwe	Malipati
C. tuttifrutti	A7GMAZW	submitted	submitted	submitted	-	Zimbabwe	Malipati
C. tuttifrutti	A7HMAZW	submitted	submitted	submitted	-	Zimbabwe	Malipati
C. tuttifrutti	ZA.PR.06	submitted	submitted	submitted	-	South Africa	Pretoria
C. zuluensis	B2AMAMG	submitted	submitted	submitted	submitted	Madagascar	Masoandro
C. zuluensis	B2CMAMG	submitted	submitted	submitted	-	Madagascar	Masoandro
C. zuluensis	B3CMAMG	submitted	submitted	submitted	-	Madagascar	Masoandro
C. zuluensis	B3DMAMG	-	-	-	submitted	Madagascar	Masoandro
C. zuluensis	F1DMKKEN	submitted	submitted	submitted	-	Kenya	Munukathi
C. zuluensis	ZA.6	submitted	submitted	submitted	submitted	South Africa	Port Elizabeth
C. zuluensis	ZA.7	submitted	submitted	submitted	-	South Africa	Port Elizabeth

Author contributions

M.T.B. and C.G. designed the study. K.L. and M.F. contributed the collection and identification of *Culicoides*. M.T.B., K.H., B.M. and C.G. analysis the data. K.L., B.M., K.H., G.V., A.G.F., T.B., L.G. G.G. and J.B. contributed to the manuscript firstly written by M.T.B. and C.G. All authors read and commented the final manuscript version.

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Chapitre 3. Amélioration des connaissances sur le comportement trophique des espèces d'intérêt

Le cycle biologique des *Culicoides* implique une étape cruciale pour les femelles hématophages. Elles doivent rechercher un hôte vertébré afin d'obtenir le repas de sang obligatoire pour la maturation des œufs. On définit la préférence trophique d'une espèce hématophage comme la tendance préférentielle à sélectionner un certain type d'hôte dans une classe de vertébrés (Clements, 1999). On distingue les espèces à large spectre d'hôte dites opportunistes, voire mammophiles (piquant préférentiellement les mammifères) ou ornithophiles (piquant préférentiellement les oiseaux), des espèces spécialisées ayant un spectre d'hôte plus spécifique. Chez les *Culicoides*, les études sur les préférences trophiques ne sont pas très approfondies aussi bien en région Paléarctique qu'Afrotropicale (Fall *et al.*, 2015b; Fall *et al.*, 2015c; Garros *et al.*, 2011; Lassen *et al.*, 2012; Viennet *et al.*, 2012).

Des approches directes et indirectes ont été menées pour caractériser le comportement trophique des espèces d'intérêt ou les plus abondantes. Les approches directes permettent de comparer les abondances collectées avec des pièges à appât avec différents types d'hôtes (Fall *et al.*, 2015a; Fall *et al.*, 2015b; Viennet *et al.*, 2011; Viennet *et al.*, 2012). Elles sont logistiquement difficiles à mettre en œuvre sur le terrain. Les approches indirectes reposent sur l'identification de l'origine des repas de sang de femelles capturées gorgées (Garros *et al.*, 2011; Lassen *et al.*, 2012; Ninio *et al.*, 2010). Ces deux approches ne tiennent pas compte de la répartition spatiale et de la diversité des hôtes vertébrés potentiels présents sur le site d'étude. L'interprétation du comportement trophique et la caractérisation de la préférence trophique sont donc toujours compliquées par cette absence de représentation globale de la disponibilité d'hôtes.

Dans ce chapitre, nous présentons une étude originale et méthodologiquement innovante pour caractériser le comportement trophique de trois espèces d'intérêt présentes dans la zone des Niayes au Sénégal, *C. imicola*, *C. kingi* et *C. oxystoma*. L'approche s'inspire d'une méthode utilisée en agronomie pour évaluer le spectre de plantes hôtes d'espèces ravageurs (Carrière *et al.*, 2006; Sivakoff *et al.*, 2013). L'objectif de ce travail était d'identifier moléculairement l'origine du repas de sang de femelles gorgées de trois espèces de *Culicoides* et de mettre ces observations en corrélation avec la distribution spatiale des hôtes mammifères potentiels présents sur le site d'étude (bovin, cheval, chèvre et mouton)

dans un rayon de 2 000 mètres. Ces résultats font l'objet de l'article 2 publié dans la revue ***Acta Tropica***.

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Foraging range of arthropods with veterinary interest: New insights for Afrotropical *Culicoides* biting midges (Diptera: Ceratopogonidae) using the ring method

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ABSTRACT

The identification of blood meal source of arthropod vector species contributes to the understanding of host-vector-pathogen interactions. The aim of the current work was to identify blood meal source in *Culicoides* biting midge species, biological vectors of internationally important arboviruses of livestock and equids, using a new ecological approach. We examined the correlation between blood meal source identified in engorged *Culicoides* females collected in a suction light trap and the available vertebrate hosts along four rings (200, 500, 1000 and 2000 m) centered at the trap site and described the foraging range of the three main vector species of veterinary interest present in the study area, *Culicoides imicola*, *Culicoides kingi* and *Culicoides oxystoma*. The study was performed in four sites localized in the Niayes region of Senegal (West Africa) where recent outbreaks of African horse sickness occurred. Blood meal source identification was carried out by species-specific multiplex PCRs with genomic DNA extracted from the abdomen of engorged females collected during nine night collections for twenty-six collections. The four most abundant hosts present in the studied area (horse, cattle, goat and sheep) were surveyed in each ring zone. The blood meal source varied according to *Culicoides* species and host availability in each site. *C. oxystoma* and *C. imicola* females mainly fed on horses readily available at 200 m maximum from the trap location whereas females of *C. kingi* fed mainly on cattle, at variable distances from the traps (200 to 2000 m). *C. oxystoma* may also feed on other vertebrates. We discuss the results in relation with the transmission of *Culicoides*-borne arboviruses and the species dispersion capacities.

1. Introduction

Finding a host is a crucial event in the life-history of haematophagous dipterans to obtain the mandatory blood meals for egg maturation. Feeding success on a given host relies on host preference, host availability and host defensive reactions. Host preference can be defined as the inherited tendency to select a group of vertebrate hosts (mammals *vs* birds), or a given host species (horse *vs* cattle) to feed on (Balenghien et al., 2011; Takken and Verhulst, 2013). Host choice is the result of a trade-off between the advantages of finding the optimal host and the risk of dying before blood feeding. This determines opportunistic or specialized feeding behavior (Lyimo and Ferguson, 2009). The description of host preferences and foraging behavior of species involved in pathogen transmission is important to improve our understanding of epiological cycles (Gubbins et al., 2008). Host diversity and availability may impact pathogen transmission by allowing transmission between different host species or leading to a dilution effect (Balenghien et al., 2011; Schmidt and Ostfeld, 2001).

The genus *Culicoides* Latreille (Diptera: Ceratopogonidae) encompasses around 1358 species worldwide (Borkent, 2015). Certain *Culicoides* species are known vectors of bluetongue virus (BTV) in wild and domestic ruminants and African horse sickness virus

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(AHSV) in equids, both devastating animal diseases that lead to substantial economic losses (Carpenter et al., 2013; Mellor et al., 2000). African horse sickness affects equids in Sub-Saharan Africa and mortality rates frequently exceed 90% in horses (Mellor and Hamblin, 2004). In Senegal, epizootic outbreaks of AHS occurred in 2007 in two localities (Niague, Mbao) in the Niayes area, before reaching other localities in the country (Diouf et al., 2012). These outbreaks affected 26 out of 34 departments in Senegal (Diouf et al., 2012) and caused important economic costs estimated at 1.37 million euros (Akakpo et al., 2011).

Feeding behavior of *Culicoides* species has received great attention recently in the Palearctic region (Ayllón et al., 2014; Blackwell et al., 1994; Calvo et al., 2012; Garros et al., 2011; Martinez-de la Puente et al., 2015; Ninio et al., 2010; Santiago-Alarcon et al., 2012). In the Afrotropical region, feeding behavior and host preferences of *Culicoides* species of veterinary interest are mainly described in South Africa (Meiswinkel et al., 2004; Nevill and Anderson, 1972). In Sudan, *Culicoides* kingi Austen is mainly attracted by cattle (El Sinnary et al., 1985). Previous studies in the Niayes region characterized the abundance of the five most abundant species in the vicinity of horses: *Culicoides imicola* Kieffer, *C. kingi*, *Culicoides oxystoma* Kieffer, *C. enderleini* Cornet and Brunhes and *C. nivosus* de Meillon (Diarra et al., 2014), and described a strong trophic preference for horses *vs* sheep for the first three species (Fall et al., 2015a, 2015c).

Different experimental designs have been developed in the field to analyze host feeding habits of *Culicoides* vector species and provided relevant information on host preferences of *Culicoides* species of veterinary interest in Europe (Viennet et al., 2013, 2011, 2012) and in Africa (Fall et al., 2015a,b,c). The most unbiased approach is to collect females directly on animal hosts but the small size of *Culicoides* makes it very difficult. Several authors also used animal-baited traps as for entomological investigations on West Nile fever virus (Balenghien et al., 2011; Fall et al., 2011). However, these direct methods are usually difficult to set up in the field and time consuming. Therefore, indirect approaches using serological or molecular identification assays have been developed. In most studies on trophic behavior, the host preferences are determined by examining the blood meal origin of field-collected specimens (using species-specific molecular assays or sequencing) (Garros et al., 2011; Lassen et al., 2012; Ninio et al., 2010) but feeding rates have rarely been linked quantitatively to host availability in the studied area. It is difficult to enumerate the numbers of available domestic and wild hosts in an area, and to estimate their spatial and seasonal variations (Balenghien et al., 2011). Furthermore, the collection of blood-fed *Culicoides* females is challenging especially because suction light traps do not allow the collection of large numbers of engorged females. Female *Culicoides* resting sites are largely unknown. Authors usually hypothesize that engorged females collected in a trap feed on the hosts present at the vicinity of the trap, except when no identified hosts are present at the trap location which allow inferring on dispersal and host preferences (Garros et al., 2011). Therefore, enumerating hosts in the close area of the trap site is required (Balenghien et al., 2011).

The ring method is frequently used in agricultural entomology and allows the investigation of dynamics and feeding behavior of polyphagous insect pests in relation to landscape composition and crop surfaces (Carrière et al., 2006; Sivakoff et al., 2013). In this study, we applied this ecological method to *C. imicola*, *C. kingi* and *C. oxystoma* in order to determine the foraging range of these three *Culicoides* species of veterinary interest abundant in the Niayes areas of Senegal. Indeed, *C. imicola* is regarded as the most important and proven vector species of Orbivirus of livestock diseases. This species has been associated with BTV (Venter et al., 2006, 1998) and AHSV (Paweska et al., 2003; Venter et al., 2000, 2009; Venter and Paweska, 2007). *C. oxystoma* is a well-known vector of bovine arboviruses such as Akabane virus (Oem et al., 2013; Yanase et al., 2005) and is suspected of being vector of Epizootic Haemorragic Disease Virus in Israel (Morag et al., 2012). *C. kingi* is involved in the transmission of *Onchocerca gutturosa*, a widespread parasite of Sudanese cattle (Sinnary and Hussein, 1980). Breeding sites of these species are generally moist soil more or less enriched with organic matter for *C. imicola* (Meiswinkel et al., 2004), alkaline wet mud for *C. kingi* (Cornet, 1969) and aquatic or semiaquatic environment, such as paddy field, stream edge and pond margin for *C. oxystoma* (Yanase et al., 2013).

We investigated the correlation between blood meal source identified for the *Culicoides* females collected in a suction light trap, and the relative abundance of vertebrate hosts along various rings around the trap location with a peculiar interest for sheep, goat, cattle and especially horse because of the recent AHS outbreak in the area. Host survey was facilitated by the absence of wild ruminants and the absence of animal movements in the area.

2. Material and methods

2.1. Culicoides *collection and survey of vertebrate hosts*

Adult midges were collected using a suction light trap of the OVI type (Onderstepoort Veterinary Institute, South Africa) placed at four sites (2 traps per site) in the Niayes area, Senegal (Fig. 1) for nine night collections (Table 1). The Niayes area is a coastal band 25–30 km wide stretching over 180 km from Dakar to the southern tip of the Senegal River Delta. The climate is oceanic, typically warm and humid with strong, relatively constant winds. The total annual rainfall is 300–350 mm/year, with a maximum in September. Mean daily minimum and maximum temperatures range from 18 °C to 31 °C with an annual mean temperature of 27 °C (more details are provided in Faye et al. (1995)).

All collected specimens were preserved in 90% ethanol, identified and sexed under a stereomicroscope using the identification keys of Cornet and Brunhes (1994), Boorman (1989) and Meiswinkel (1989). Of 43,484 specimens collected in the light traps (Table 1), only 270 fully engorged females of *C. imicola*, *C. kingi* and *C. oxystoma* were considered and used in this study (Table 3). Information on vertebrate host position was collected around each trapping site on a radius of 2000 m (Table 1). Movements of domestic ruminants/horses are very much restricted in the Niayes area. Surveys showed that the spatial and seasonal movements of vertebrate hosts are negligible in this area during the studied period. Free wild fauna is completely absent from this area.

2.2. *DNA extraction, primers design and PCR*

Genomic DNA was extracted using commercial kits (Macherey-Nagel, Germany). Host primers were selected as described in the literature (Garros et al., 2011; Kocher et al., 1989) for identifying the origin of *Culicoides* blood meals (Table 2). Molecular identification was based on the amplification of the cytochrome b region of blood DNA as described in Garros et al. (2011). A multiplex PCR was performed to separate cattle, sheep and goat, and a simplex PCR was used to identify blood meal from horse (Garros et al., 2011).

2.3. *Statistical analysis*

Host positions and host densities (cattle, goat, horse, sheep and other vertebrates) were surveyed and calculated in the four concentric rings at distances of 200 m, 500 m, 1000 m and 2000 m around the trapping site (MapInfo® software, version 7.0). Selection of vertebrate hosts by *Culicoides* species was determined by correlation (Pearson's product-moment correlation). We examined the correlation between the percentage of the identified host as

Table 1
Diversity of vertebrate hosts and *Culicoides* specimen collection per site.

Trapping site	Latitude	Longitude	Vertebrate animals recorded in each ring (r) from the trapping site					Culicoides collected	
			r = 200 m	r = 500 m	r = 1000 m	r = 2000 m	Survey date	Total specimens of all species	Collection date
Parc de Hann[a]	14°72′83″N	17°42′98″W	80 horses, 28 sheep and 2 dogs	80 horses, 116 sheep and 4 dogs	80 horses, 2 cows, 8 goats, 253 sheep, 313 poultry, 11 lions, 1 tiger, 3 hyenas, 5 jackals, 6 antelopes, 21 crocodiles, 9 turtles, 7 pythons and 2 pumas	80 horses, 2 cows, 275 sheep, 8 goats, 313 poultry, 11 lions, 1 tiger, 3 hyenas, 5 jackals, 6 antelopes, 21 crocodiles, 9 turtles, 7 pythons and 2 pumas	26 and 30 April 2012	1272	17–18 May 2012 18–19 May 2012 17–18 June 2012 18–19 June 2012
Mbao	14°74′67″N	17°33′27″W	32 horses, 1 dog, 2 poultry and 3 goats	35 horses, 1 dog, 28 sheep, 3 goats and 127 poultry	38 horses, 1 dog, 3 goats, 181 sheep, 552 poultry and 8 rabbits	38 horses, 1 dog, 9 cows, 207 sheep, 7 goats, 566 poultry and 8 rabbits	17 and 18 May 2012	7847	17–18 May 2012 18–19 May 2012 17–18 June 2012 18–19 June 2012
Niague	14°82′34″N	17°24′99″W	43 horses, 4 cows, 6 sheep, 1309 poultry and 1 dog	117 horses, 4 cows, 6 sheep, 7089 poultry and 1 dog	117 horses, 256 cows, 191 goats, 60 sheep, 12731 poultry, 10 dogs and 9 rabbits	117 horses, 426 cows, 86 sheep, 320 goats, 13059 poultry, 12 dogs and 9 rabbits	17, 18 and 19 July 2012	32,452	18–19 July 2012
Thiès	14°79′40″N	16°95′00″W	24 horses, 2 sheep, 50 poultry and 14 rabbits	24 horses, 27 sheep, 118 poultry, 14 rabbits and 2 dogs	24 horses, 41 cows, 75 goats, 123 sheep, 299 poultry, 7 dogs and 22 rabbits	24 horses, 41 cows, 142 sheep, 75 goats, 314 poultry, 7 dogs and 22 rabbits	20 and 21 May 2012	1913	20–21 May 2012 21–22 May 2012 20–21 June 2012 21–22 June 2012

[a] Zoo is located about 800 m from the trap.

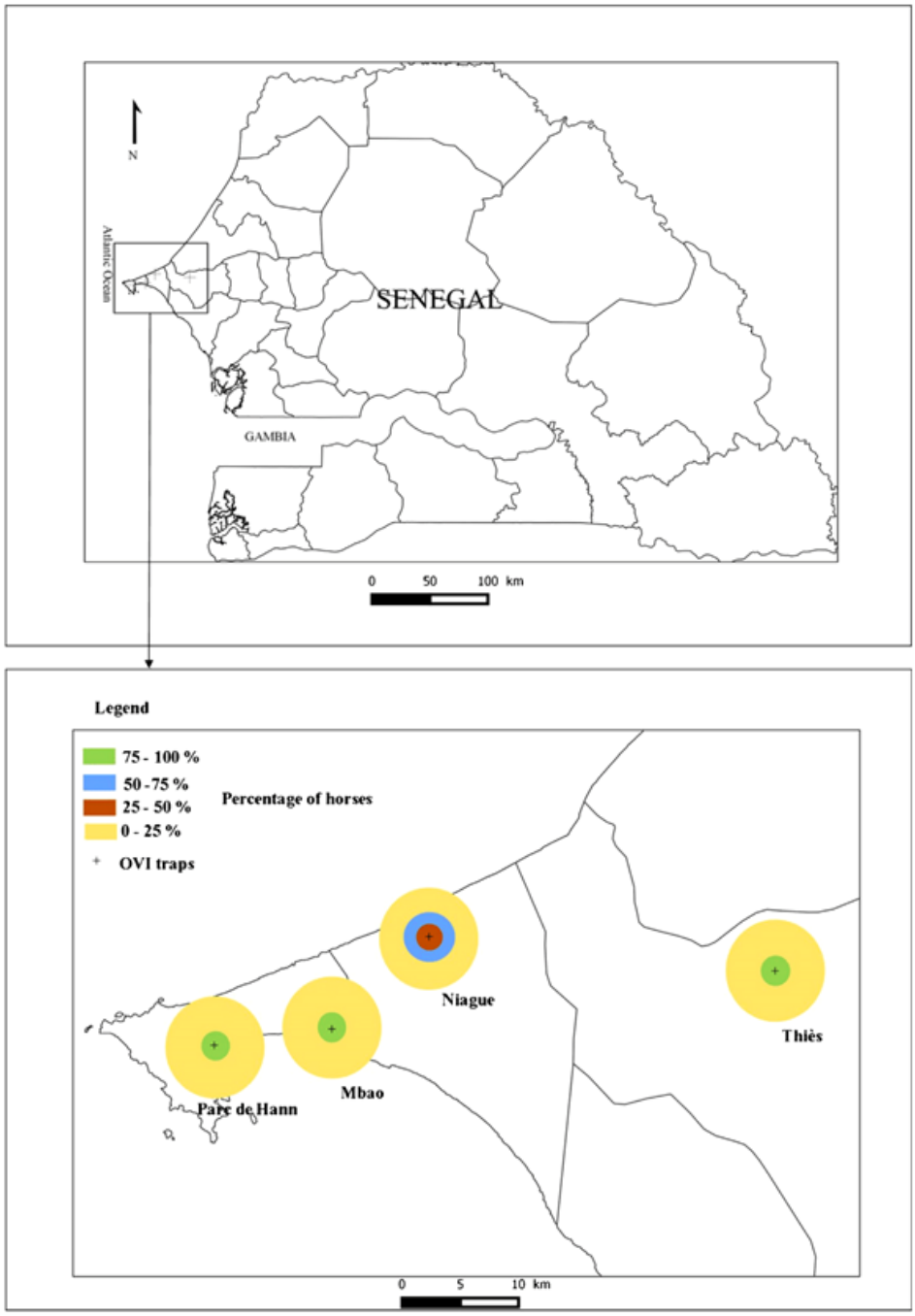

Fig. 1. Location of the four study sites in the Niayes area of Senegal. The circles present the relative abundance of horses around the trap site.

blood meal source and the percentage of availability of this host within each ring corrected by their weight. The average weight of hosts were estimated as follows: pigeon (0.5 kg), rabbit (4 kg), jackal (13 kg), dog (15 kg), turtle (20 kg), goat (30 kg), antelope (40 kg), puma (40 kg), sheep (40 kg), hyena (60 kg), ostrich (60 kg), crocodile (100 kg), python (100 kg), lion (160 kg), tiger (200 kg), cattle (400 kg), horse (450 kg) (Data obtained at the veterinary services and livestock agents).

Table 2
Primer set used for the identification of blood meal origin in *Culicoides* abdomens.

Primers	Primer sequence (5′ → 3′)	Length (pb)
VERT-F (Forward primer vertebrate)	CCATCCAACATCTCAGCATGATGAAA	
VERT-R (Reverse primer vertebrate)	GCCCCTCAGAATGATATTTGTCCTCA	357
Forward primer UNIV 3	TTTTTTTTTTTTCGVTCHATYCCHAAYAAACTAGG	
EQUUS-R (*Equus caballus*)	TACGTATGGGTGTTCCACTGGC	208
Forward primer UNIV 2	TGAGGACAAATATCATTYTGAGGRGC	
BOS-R (*Bos taurus*)	TAAGATGTCCTTAATGGTATAGTAG	287
CAPRA-R (*Caprus hircus*)	TTAGAACAAGAATTAGTAGCATGGCG	313
OVIS-R (*Ovis aries*)	GGCGTGAATAGTACTAGTAGCATGAGGATGA	336

Table 3
Number of engorged females (N_{eng}) relative to the total number of females collected (N_T).

	C. kingi (N_{eng}/N_T)	*C. imicola* (N_{eng}/N_T)	*C. oxystoma* (N_{eng}/N_T)	Total
Parc de Hann	0/1	2/14	56/546	58/561
Mbao	10/3498	17/1177	42/1798	69/6473
Niague	53/30,292	4/548	2/355	59/31,195
Thiès	0/16	33/336	51/1328	84/1680
Total	63/33,807	56/2075	151/4027	270/39,909

3. Results

3.1. Culicoides *collection*

For nine night collections at 4 sites (twenty-six collections), 43,484 specimens of *Culicoides* species were collected (Table 1). Of these specimens, 39,909 were represented by *C. kingi*, *C. imicola* and *C. oxystoma*. A total of 270 engorged females belonging to these three species were obtained (Table 3).

3.2. *Blood meal identifications*

Identification of blood meal sources was successful for 155 out of 270 blood-fed females (57%), of which 110 were identified as horse, 24 as cattle and 21 on other vertebrates (Table 4). No blood meals were identified on sheep or goat.

3.3. *Correlation between survey data of vertebrate hosts and blood meals identified*

Fig. 2 shows the blood meal source identified at the 4 sites in relation to the composition of host species corrected by their weight along the transect. Different correlation coefficients were found according to *Culicoides* species (Fig. 3). For *C. imicola*, the highest correlation was observed in the lowest range (<200 m) ($r^2 = 0.998$, $t = 35.118$, $df = 3$, $p < 10^{-3}$). For *C. kingi*, the correlation was high from the 1000 to 2000 m range ($r^2 = 0.95$, $t = 5.299$, $df = 3$, $p = 0.013$). For *C. oxystoma*, similar correlation coefficients were found at all ranges in Parc de Hann ($r^2 = 0.543$, $t = 1.122$, $df = 3$, $p = 0.343$) and Mbao ($r^2 = 0.957$, $t = 88.77$, $df = 3$, $p = 0.014$). In Thiès, the highest correlation was observed for a range below 200 m ($r^2 = 0.999$, $t = 227.25$, $df = 3$, $p < 10^{-3}$), as also described for *C. imicola*.

4. Discussion

The characterization of host preferences of vector species is an important key to understand the transmission of vector-borne pathogens. In this study, we assessed the feeding preferences and foraging range of *Culicoides* species of veterinary interest in Senegal using the ring method and molecular blood meal identification.

The recent developments in molecular biology allow reliable and effective identification of blood meal source of field-collected insect specimens, as previously performed on mosquitoes (Fall et al., 2012; Gokool et al., 1993; Kent and Norris, 2005; Ngo and Kramer, 2003) and on *Culicoides* (Ayllón et al., 2014; Elbers and Meiswinkel, 2014; Garros et al., 2011; Martinez-de la Puente et al., 2015). In this study, blood meals were identified by amplification of the cytochrome b region of genomic DNA extracted from abdomen of engorged females of *Culicoides* species using multiplex and simplex PCRs. However, blood meal source for 43% of engorged females failed to be identified. This unexpected loss of efficiency is probably due to the degradation of host DNA present in the blood meal. Indeed, the digestion processes quickly denature the genomic DNA of blood meal making identification difficult or impossible (Kent and Norris, 2005; Oshaghi et al., 2005). Moreover, the long-term conservation of specimens in alcohol (between 2 to 3 years) may also have contributed to degrade the genomic DNA of blood ingested by females. One limitation is the small number of engorged females collected. Therefore, sufficient numbers of identified blood meals to permit statistical analysis of host preference and foraging ratios were only obtained for certain sites and species.

In contrast to previous studies on feeding preferences of *Culicoides*, *i.e.* using indirect approaches (serological and molecular analyzes from the abdomens of females collected), our study has taken into account the diversity and availability of potential hosts around the sampling site. In this study, blood meal source varied according to *Culicoides* species and host availability. Blood meals were mainly taken around the trapping site (<200 m) for *C. imicola* and *C. oxystoma* in one site whereas *C. kingi* seems to present a different foraging strategy, with blood meal taken at an average of 2000 m. Potential breeding sites of these species are located in the vicinity of the trap sites, except those of *C. kingi*. Breeding sites of this species is the edge of the Lac Rose located at 2.5 km from the trap site of Niague (unpublished data).

Among vertebrate host studied, horse was the principal species attacked by *Culicoides* (71% of blood meal) and no blood meals were taken on sheep or goat. Although sheep and goat were present around the trapping sites, their relative abundance was very low in comparison to horse (horse = 73%, sheep = 9% and goat = 1% for all sites). Studies using direct approaches (*i.e.* to collect females directly on animal hosts) shown that *Culicoides* species are more attracted to horses than to sheep in the same area (Fall et al., 2015b, 2015c).

Local populations of *C. oxystoma* mainly fed on horses in the vicinity of the trapping site. In Parc de Hann, only 48% of *C. oxystoma* individuals fed on horses readily available at 200 m whereas 52% fed on other vertebrates (different from sheep, goats and cattle). This high rate of non-identified blood meal (52%) is probably related to

Table 4
Blood meal identification in *Culicoides* females collected from four sites in the Niayes area in Senegal.

Sites	*Culicoides* species	Cattle	Goat	Sheep	Horse	Other vertebrates	Total
Parc de Hann	*C. oxystoma*	–	–	–	11	12	23
Mbao	*C. imicola*	–	–	–	3	–	45
	C. kingi	–			5	–	
	C. oxystoma	–			37	–	
Niague	*C. kingi*	23	–	–	9	9	41
Thiès	*C. imicola*	1	–	–	22	–	46
	C. oxystoma	–	–	–	23	–	
Total		24			110	21	155

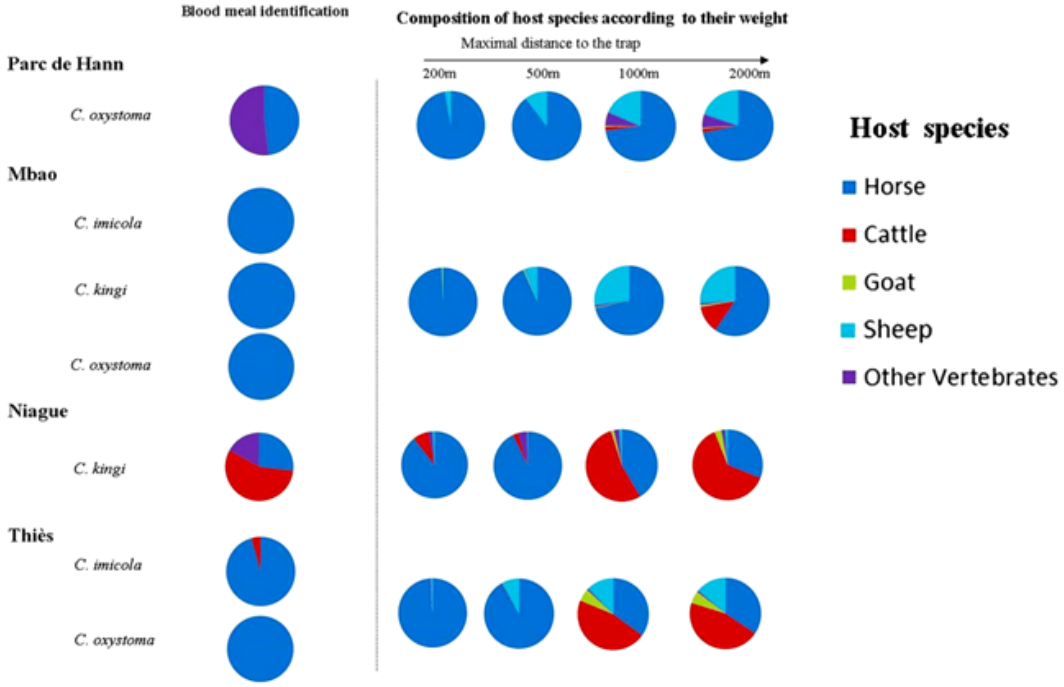

Fig. 2. Identified blood meals and composition of host species corrected by their weight at different distances from the trapping sites.

the presence of a zoo located at 800 m from the trap position. *C. oxystoma* showed a wide foraging range.

Considering *C. imicola*, horse was the favorite host in this study and this species mainly fed in the vicinity of the trapping sites in Thiès. Previous studies showed *C. imicola* is attracted by horses (Fall et al., 2015a, 2015c; Meiswinkel et al., 2004; Nevill and Anderson, 1972). Our results demonstrated a strong host preference for horses (96%) but *C. imicola* is also able to feed on cattle at nearly 1 km. This observation illustrates that an engorged female of *C. imicola* can to be up to 1 km after a blood meal whether by active dispersal.

Our results show that *C. kingi* presents an opportunistic feeding behavior with a diverse range of vertebrate hosts and the best correlation with host availability was observed at 2000 m in the trapping site of Niague. This population of *C. kingi* seemed to be attracted by cattle in this site, as it was also the case in another study performed in Sudan using cow-baited traps (El Sinnary et al., 1985).

In Niague, cattle were more abundant after 1000 m than around the trapping site. The relative abundance of cattle after 1000 m was more correlated with the blood meals composition of *C. kingi* leading to suspect an active dispersal of *C. kingi* within a distance of 1000–2000 m. This foraging behavior leading to flight around 2000 m may imply important cost benefit trade-off in the bio-ecology of this species. However, *C. kingi* could also have fed on cattle within the ring of 200 m from of the trapping site thanks to a strong specific selection of this species.

Generally, the active dispersion of *Culicoides* specimens is assumed to be short, average distance of more or less than 2 km from their breeding sites, as for *Culicoides obsoletus* (Kluiters et al., 2015) and *Culicoides* pulicaris (Kirkeby et al., 2013) in the Palearctic region. This has never been investigated for Afrotropical species. Beside the active dispersion, the movement of *Culicoides* can be aided by the wind. This passive dispersion could be several kilometers and plays an important role in the spatial dynamics of epidemics of *Culicoides*-borne diseases essentially the bluetongue (Braverman and Chechik, 1996; Eagles et al., 2014; Sellers et al., 1979).

The originality of our study relies on the use of a new method in medical and veterinary entomology applied to insect vectors allowing studying the foraging range of *Culicoides* species. The foraging behavior of the studied species varied with host availability and their foraging. It was found that *C. oxystoma* and *C. imicola* mainly fed on horse unlike *C. kingi* which appear to favor cattle but can also feed on horses in the absence of cattle. None of these species fed on goats and sheep although some of these hosts were available near trapping sites. These behavioral results reinforce the impor-

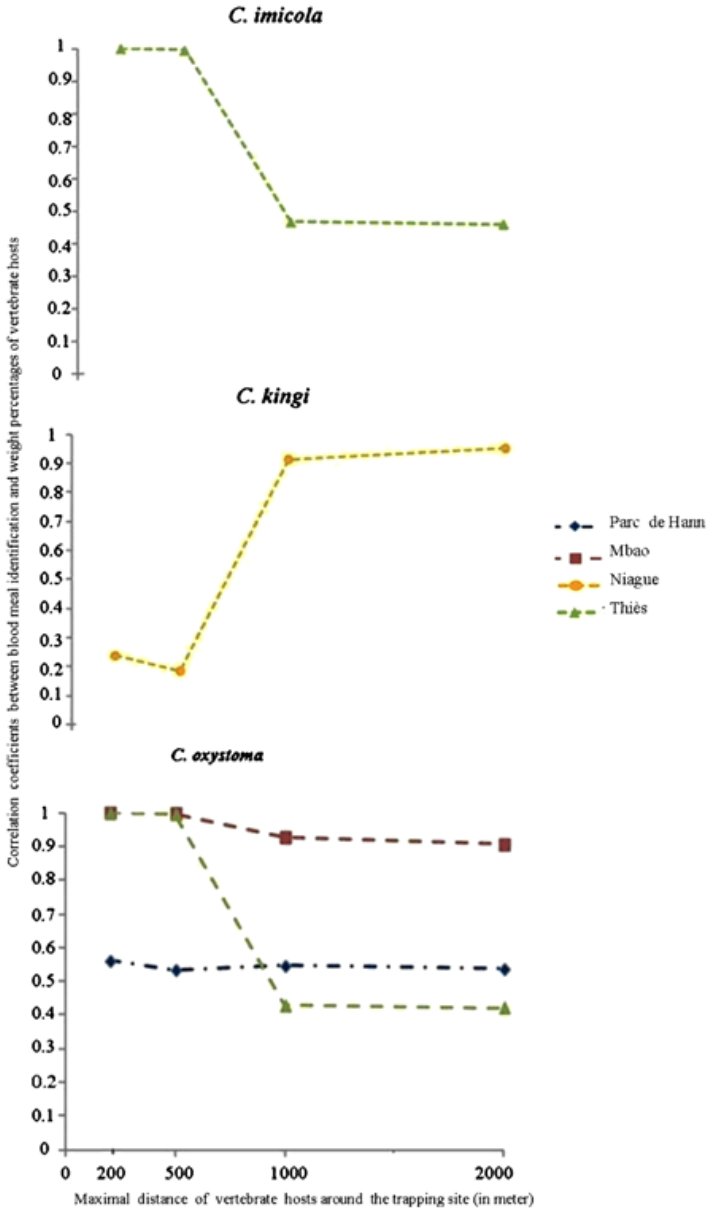

Fig. 3. Correlation between host selection by *Culicoides* species and vertebrate host availability.

tance of these species as putative vector species for AHSV in the Niayes region although their formal vector competence has still not been investigated. It would be interesting to replicate this study in controlled conditions in at different times to evaluate seasonal variations of the foraging range and in sites where horses are scarce compared to other potential hosts to evaluate the relative importance of innate preferences for these *Culicoides* species. This will improve our understanding and prevention of the transmission of *Culicoides*-borne viruses.

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Author contributions

Conceived and designed the experiments: MTB CG AGF MTS JB. Contributed the experiments: MTB CG MF LG IM MD. Analyzed the data: MTB CG TB JB. Wrote the first draft of the manuscript: MTB CG MF LG AGF TB MTS GG JB. All authors revised and approved the final version of the manuscript.

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Chapitre 4. Ecologie larvaire et outils moléculaires d'aide à l'identification : application à la caractérisation des habitats larvaires et à la dynamique des populations d'immatures au Sénégal

L'écologie larvaire des *Culicoides* est un champ de recherche peu exploré. Ceci s'explique par la difficulté de collecter les stades immatures et la quasi-impossibilité d'identifier ces stades du fait du manque de clés d'identification morphologique. Le manque de données caractérisant les gîtes d'oviposition ou de développement des immatures ainsi que des déterminants biotiques et abiotiques expliquant la dynamique des populations d'immatures limite le développement de méthodes de lutte efficace. Certaines espèces comme *C. tuttifrutti* sont décrites comme ayant un habitat larvaire très spécifique (figues de Barbarie en décomposition). D'autres espèces comme *C. imicola* peuvent se développer dans plusieurs types d'habitat (à bord de bergeries, des étangs ou des mares). Enfin, plusieurs espèces de *Culicoides* peuvent partager le même habitat larvaire (Dipeolu et Ogunrinade, 1976; Jenkins et Young, 2010; Labuschagne, 2016; Lubega et Khamala, 1976; Meiswinkel, 1987; 1992; Nevill, 1967; Nevill *et al.*, 2007). Malgré tout, ces données restent fragmentaires, et limitées à certaines espèces dans des environnements spécifiques aux ruminants domestiques et/ou en Afrique Australe.

L'étude présentée au chapitre 2 nous a permis d'identifier une grande partie de la diversité Afrotropicale d'intérêt vétérinaire pour le genre *Culicoides* et de produire une base de données de séquences moléculaires de référence (séquences barcodes) à partir de spécimens adultes identifiés morphologiquement. Peu d'études ont utilisé l'identification moléculaire des stades immatures principalement à cause de l'absence de bases de données de référence de la diversité étudiée (Yanase *et al.*, 2013). Les travaux d'écologie larvaire présentés dans ce chapitre combinent pour la première fois en région Afrotropicale un suivi classique de populations d'immatures et une approche d'identification moléculaire. Dans un premier temps, nous avons investigué et décrit les habitats larvaires des espèces de *Culicoides* d'intérêt vétérinaire dans la zone des Niayes au Sénégal dans un environnement équin (article 3). Puis, nous avons réalisé une librairie de séquences barcodes pour la diversité et utiliser cette base de données comme outil d'aide à l'identification des larves de *Culicoides*.

Les résultats de ces travaux de recherche sont présentés dans l'article 3 publié dans la revue ***Parasites & Vectors***, et dans les articles 4 et 5 en **cours de préparation**.

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Parasites & Vectors

SHORT REPORT Open Access

Insight on the larval habitat of Afrotropical *Culicoides* Latreille (Diptera: Ceratopogonidae) in the Niayes area of Senegal, West Africa

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Abstract

Background: Certain biting midges species of the genus *Culicoides* (Diptera: Ceratopogonidae) are vectors of virus to livestock worldwide. *Culicoides* larval ecology has remained overlooked because of difficulties to identify breeding sites, methodological constraints to collect samples and lack of morphological tools to identify field-collected individuals to the species level. After the 2007 unforeseen outbreaks of African horse sickness virus (AHSV) in Senegal (West Africa), there is a need to identify suitable and productive larval habitats in horse farms for the main *Culicoides* species to evaluate the implementation of vector control measures or preventive actions.

Methods: We investigate twelve putative larval habitats (habitat types) of *Culicoides* inside and outside of three horse farms in the Niayes area of Senegal using a combination of flotation and emergence methods during four collection sessions.

Results: Among the three studied horse farms, three habitat types were found positive for *Culicoides* larvae: pond edge, lake edge and puddle edge. A total of 1420 *Culicoides* individuals (519♂/901♀) belonging to ten species emerged from the substrate samples. *Culicoides oxystoma* (40 %), *C. similis* (25 %) and *C. nivosus* (24 %) were the most abundant species and emerged from the three habitat types while *C. kingi* (5 %) was only retrieved from lake edges and one male emerged from puddle edge. *Culicoides imicola* (1.7 %) was found in low numbers and retrieved only from pond and puddle edges.

Conclusions: Larval habitats identified were not species-specific. All positive larval habitats were found outside the horse farms. This study provides original baseline information on larval habitats of *Culicoides* species in Senegal in an area endemic for AHSV, in particular for species of interest in animal health. These data will serve as a point of reference for future investigations on larval ecology and larval control measures.

Keywords: *Culicoides*, Larval habitats, Flotation technique, Senegal, African horse sickness

Abbreviations: BTV, Bluetongue virus; AHSV, African horse sickness virus; AHS, African horse sickness

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Background

Biting midge species of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) comprise around 1358 described species distributed worldwide [1]. Certain *Culicoides* species are known as the biological or putative vectors of viruses of domestic and wild ruminants as well as horses, such as the Schmallenberg virus (SBV), Akabane virus (AKAV), Bluetongue (BT) virus, epizootic haemorrhagic disease (EHD) virus and African horse sickness (AHS) virus [2, 3]. Vector control strategies for *Culicoides* spp. are needed. Indeed, vector control aims at reducing density of *Culicoides* populations at adult and larval stages, to limit host-vector contacts and hence decrease virus transmission [4, 5]. Among putative strategies, biological, chemical or environmental control of immature stages have been overlooked principally because oviposition behavior, breeding sites, larval habitats and factors regulating immature abundance are mostly unknown for *Culicoides* vector species. Baseline data on larval habitats of main vector species worldwide are needed to have a better understanding of their ecology and to provide new insights to the development of efficient vector control measures [6–9].

Culicoides spp. larval habitats are usually defined as humid rich and enriched in animal or vegetal organic matter, and may cover a wide range of natural and artificial substrates. Indeed, many larval habitats are described worldwide, including freshwater marshes and swamps, shallow margins of ponds, streams and rivers, bogs and peat lands, beaches, around leaking irrigation pipes and water troughs, tree holes and other natural cavities in rotting wood, waterlogged pastures, animal manure, rotting fallen fruits, highly alkaline or saline inland pools and animal dung [10–20].

Species diversity of *Culicoides* could be locally high [21–23], but it is admitted that only a limited number of species are able to transmit AHSV or BTV [2]. Consequently, larval ecology of only abundant vector species is described for livestock-related areas in some areas where the species occur [13, 23–25]. The main BTV vector in northern America, *C. sonorensis*is associated with edges of wastewater and polluted ponds on farms, but other aquatic sources (irrigation runoff in pasture, puddles, trough spillover) have also been found as high productive habitats [4, 23, 26]. In the Oriental and Australasian regions, *C. brevitarsis* larvae are mainly found associated with cattle dung [27, 28] unlike those of *C. oxystoma* which are found in aquatic and semi-aquatic habitats, such as paddy fields, stream edges, pond margins or estuary [28–30]. Larvae of the BTV vector species belonging to the subgenus *Avaritia* in the Palaearctic region (*C. obsoletus*, *C. scoticus*, *C. dewulfi*, *C. chiopterus* and *C. dewulfi*) are usually mentioned in the literature associated with cattle dung or cattle/sheep farm environment [13, 25, 31, 32], whereas *C. obsoletus* and *C. scoticus* could occupy a wide range of habitats inside and outside farm buildings [13, 32]. *Culicoides chiopterus* and *C. dewulfi* showed preferences for high soil moisture [33] and are referred as cattle dung breeders [34].

Certain species, e.g. *C. imicola*, have a wide range of larval habitats. Initially, larvae of *C. imicola* (affiliated to the subgenus *Avaritia*) are reported in permanent moist grassed margins of streams, furrows where grass is kept short by grazing animals in its southern historical distribution range (i.e. in South Africa) [17]. In northern Sardinia, an island in the Mediterranean, larval habitats of *C. imicola* are muddy habitats, not waterlogged, above unvegetated pond margins [24], which matches the first observations of *C. imicola* larval habitats in South Africa [18]. In Israel, Braverman et al. [11] described abundant larval populations in rich mixture of organic matter and water saturated soil, and concluded that this substrate was the favorable habitat of *C. imicola* (named by its synonym name *C. pallidipennis* in the article). Other descriptions of *C. imicola* larval habitats are detailed from Kenya [15], Nigeria [12] and Rhodesia (equivalent in territorial terms to modern Zimbabwe) [35], but suspected taxonomic uncertainty or misidentification [17] made the larval habitat descriptions doubtful for *C. imicola*. The anecdotal record of *C. imicola* by Nevill [36] breeding in cattle dung is also considered by Meiswinkel a misidentification [17]. Therefore, in the Afrotropical region, favorable larval habitats of *C. imicola*, the main vector species of AHSV and BTV, are only well described from South Africa. Apart from *C. imicola*, larval habitats of other Afrotropical species were investigated mostly in South Africa [14, 16, 18, 19], Zimbabwe [35] Nigeria [12] and Kenya [15]. These larval habitats can be grouped into four main types: (i) moist soil enriched greater or lesser in organic matter (decomposing plant matter, varying from intact material to humus, or of decomposed dung, such as is often found on irrigated pastures) with a great diversity of associated species [12, 15, 16, 18]; (ii) tree holes and other natural cavities in rotting wood, with often rare species such as *C. accraensis*, *C. inornatipennis*, *C. clarkei*, *C. confusus*, *C. eriodendroni*, *C. nigripennis*, C. *olyslageri* and *C. punctithorax* [16, 18]; (iii) dung pats of large herbivores such as African buffalo and cattle; and (iv) rotting fallen fruits of the sausage tree. The latter two larval habitat types are used respectively by *C. bolitinos* [16, 17] and *C. tuttifrutti* [16]. Overall, for the Afrotropical region, data on *Culicoides* larval habitats are scarce and mostly limited to southern Africa. Moreover, most of the studies have described favorable larval habitats in bovine environment areas [15–17, 35]. Despite the importance of AHSV in the African region, no larval habitats of *Culicoides* spp. are described in horse-surrounding ecosystems and no species are associated with horse dung.

Immature stages are localized at the substrate surface [37, 38]. Different techniques were used to investigate *Culicoides* spp. larval habitats and to determine the presence/absence and abundance of *Culicoides* larvae: sampling larvae from the substrate, emergence traps in the field and emergence pots. Larval collection methodology has been described and reviewed in [39] and sugar flotation provides the most effective results with low larval mortality [34]. Emergence methods are practiced by installing emergence traps directly in the field or by incubating the substrate samples in controlled environment with optimal conditions [12, 14, 15]. For larval ecology studies, a combination of both methods is necessary because no identification keys for immature stages exist for *Culicoides* spp. Recently, molecular identification of *Culicoides* larvae has been used through barcode sequences [28, 40]. This approach is powerful but is only applicable if the species diversity in a specific area has already been barcoded.

In Senegal, 53 species of *Culicoides* are recorded including species proven or suspected biological vectors of viruses of interest in animal health such as *C. imicola*, *C. bolitinos*, *C. kingi* and *C. oxystoma* [21, 22, 41, 42]. The country faced outbreaks of African horse sickness (AHS) in 2007 which caused the death of 1169 horses and considerable economic losses, estimated to 1.4 million euros [43, 44].

This study was designed to provide baseline information on larval habitats of Afrotropical *Culicoides* spp., in particular for species proven or suspected biological vectors of viruses in three horse farms in a known AHS-endemic region in Senegal. Identifying larval habitats in this area may serve as a point of reference for future investigations on larval ecology and larval control measures as already investigated in some regions [5, 7, 45].

Methods

Study sites

The study was conducted in three horse farms affected by the 2007 AHS epizootic in the Niayes area in the vicinity of Dakar and Thiès, Senegal (Fig. 1) (see references [21, 42] for study region description): (**a**) Horse farm of Mbao (latitude: 14.7467, longitude: -17.3327) is a riding centre with 32 horses surrounded by a protected forested area. (**b**) Horse farm of Niague (latitude:14.8234, longitude: -17.2499) is a riding centre with 30 horses, 1 donkey and less than 10 cows or sheep, surrounded by a market-gardening area. (**c**) Horse farm of Pout (latitude: 14.7665, longitude: -17.0357) is a modern farm located

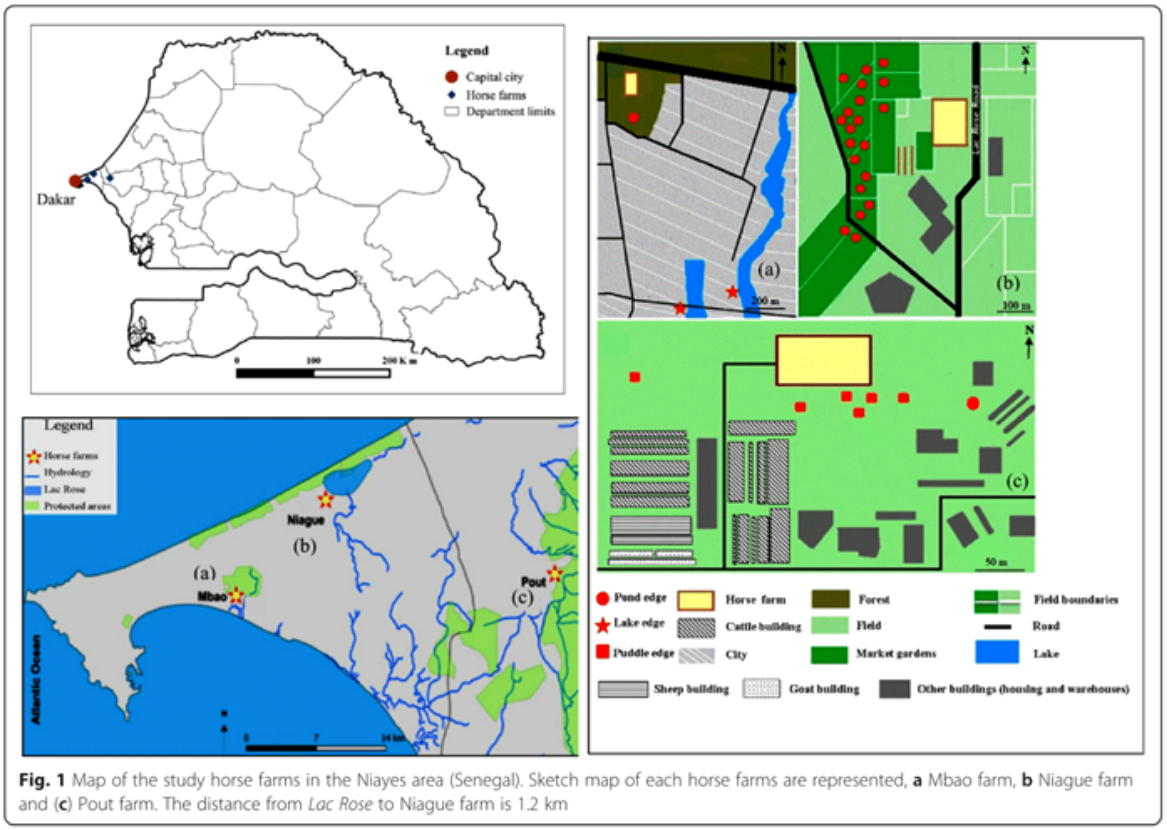

Fig. 1 Map of the study horse farms in the Niayes area (Senegal). Sketch map of each horse farms are represented, **a** Mbao farm, **b** Niague farm and (**c**) Pout farm. The distance from *Lac Rose* to Niague farm is 1.2 km

in a rural environment. The latter site owns a wide range of animals in high numbers: 20 equids, 1700 sheep, 240 goats and 1600 cows or buffaloes. In each horse farm, dung and manure in animal buildings and boxes were removed every day and accumulated outside horse farms before being used for field spreading by local farmers. Previous entomological monitoring conducted in the three horse farms revealed high abundance and large diversity of *Culicoides* spp. [21, 22].

Larval habitat classification, sample collection strategy and monitoring

We have defined and described twelve putative larval habitats (habitat types) inside and outside horse farms with reference to the literature to match previous classifications [31] (Figs. 1 and 2). At each horse farm, all the twelve defined putative larval habitat types were investigated at several sampling sites if available (Table 1).

Samples were collected in September-October 2014 during four collection sessions (16th September, 30th September, 15th October and 30th October), corresponding to the end of the rainy season. For each available defined habitat type (Fig. 2, Table 1), one substrate sample of approximately 650 cm^3 was collected in the upper layer of soil surface (0–5 cm) with a trowel, filtered with a fine mesh sieve of 0.8 mm diameter and then investigated for midge larvae in the field using a direct flotation technique in saturated sugar solution (850 g/l). Presence/absence of midge larvae was used as a proxy for positive larval habitat. If no midge larvae were observed in the first

Fig. 2 Pictures of the 12 *Culicoides* larval habitats investigated. The red *arrow* indicates where samples were collected. **a** Larval habitat 1 (outdoor fresh horse manure). The manure is wet, recently deposited on a heap. The manure was previously collected in horse boxes and is a mixture of sand and horse faeces. **b** Larval habitat 2 (outdoor old horse manure). Manure is a mixture of sand and horse faeces, partially dry and localized on a heap. **c** Larval habitat 3 (inside farm along protected fence). Samples were a mixture of manure and moist organic matter. **d** Larval habitat 4 (indoor litter soil). Solid and wet litter was collected inside horse boxes. Litter is a mix of sand and horse urine. **e** Larval habitat 5 (indoor humid soil). Solid and wet litter was collected inside horse boxes, under and around troughs. **f** Larval habitat 6 (water flow). Samples were collected at the edge of a water flow. Water resulted from washing horses and contained a lot of horse hairs, soap and organic matter (mainly faeces). Soil was sandy. **g** Larval habitat 7 (pond edge). Samples were collected at the interface between water and ground. Ponds were always located outside farms, with vegetation, and are used as water reserve for irrigation. Water is present all year along and soil was muddy and sandy. **h** Larval habitat 8 (brackish river and lake edges). Samples were collected at the interface between water and sandy soil. Water is highly polluted by riverine waste and presents a green/dark colour. **i** Larval habitat 9 (puddle edges). Samples were collected at the interface between water and ground. All the puddles sampled during our study results from water leak. Water was from a drilling and therefore does not contained chlorine. Soil was muddy. **j** Larval habitat 10 (fresh cattle dung). Samples were collected from dung deposited inside cattle boxes. **k** Larval habitat 11 (cattle dung heap). Samples were collected from a large cattle dung heap near the farm. Organic matter was humid. **l** Larval habitat 12 (liquid manure). Samples were collected from a flow channel of cattle liquid manure. It was a mix of urine and faeces

Table 1 Number of flotation for each of the twelve *Culicoides* larval habitats investigated according to horse farm and collection session

	Number of flotation/Horse farm/Collection session												
	16th September			30th September			15th October			30th October			
Larval habitat	A	B	C	A	B	C	A	B	C	A	B	C	Total
1. Outdoor fresh horse manure	3	2	3	2	–	–	–	–	–	–	–	–	10
2. Outdoor old horse manure	2	5	–	2	–	–	2	2	–	–	–	–	13
3. Inside farm along protected	–	2	–	–	–	–	–	–	–	–	–	–	2
4. Indoor litter soil	7	5	3	6	–	5	2	–	4	–	–	4	36
5. Indoor humid soil	6	2	2	4	–	2	–	–	–	–	–	–	16
6. Water flow	2	–	–	–	–	–	–	–	–	–	–	–	2
7. Pond edge	1	6	–	1	–	–	1	9	–	1	5	–	24
8. Lake edge	–	–	–	1	–	–	2	–	–	2	1	–	6
9. Puddles edge	4	–	2	6	–	5	–	–	4	–	–	3	24
10. Fresh cattle dung	–	2	–	–	–	4	–	2	4	–	–	2	14
11. Cattle dung heap	–	–	–	–	–	3	–	–	2	–	–	2	7
12. Liquid manure	–	–	–	–	–	3	–	–	2	–	–	2	7
Total	25	24	10	22	–	22	7	13	16	3	6	13	161

Abbreviations: *A* horse farm of Mbao, *B* horse farm of Niague, *C* horse farm of Pout

flotation sample, a second replicate was collected if possible. For each positive flotation sample, up to three samples of approximately 125 cm^3 were collected from the 0–5 cm of soil using a trowel and placed in 200 ml plastic pots covered with a net before being transported to the laboratory (insectarium) to monitor adult emergence. Emerging pots were maintained for 21 days at a temperature of 25 ± 1 °C, relative humidity of 80 ± 10 % and a light:dark photoperiod of 12:12 h to allow the retrieval of emerging adult *Culicoides*. The surface of the substrate was sprayed every two days with demineralized water to prevent desiccation. Each day, emerging adults were collected using a mouth-operated aspirator and then preserved in 90° ethanol. *Culicoides* species identifications were done on emerged adults using a stereomicroscope (10–40×) and reference identification keys [46–48].

Results

For the three horse farms and the four collection sessions, a total of 161 flotation samples were collected (Table 1). Of these, 45 samples (28 %) were positive for *Culicoides* spp. larvae, which resulted in 135 emergence pots.

Among the 12 putative larval habitats, three larval habitats were found positive for *Culicoides* larvae using sugar flotation collection method: (**g**) pond edge, (**h**) lake edge and (**i**) puddle edges (Fig. 2). No larvae were retrieved of samples from putative larval habitats associated with horse faeces; from a mixture of organic matter and water and from putative larval habitats associated with cattle dung (Fig. 2). All of the positive sampling sites for these three larval habitats were localized outside horse farms (Fig. 1).

A total of 1420 adult *Culicoides* (519 ♂/901 ♀) belonging to 10 species emerged from the 135 substrate samples in the laboratory (Tables 2 and 3; Figs. 3, 4 and 5) together with other dipteran species belonging to the families Ceratopogonidae (genus *Forcipomyia*) and Psychodidae (data not shown).

For the three positive larval habitats, 90 % of the emerged specimens belong to three species: *C. oxystoma* ($n = 568$), *C. similis* ($n = 362$) and *C. nivosus* ($n = 341$) (Table 2). Less than 2 % of the emerged *Culicoides* were identified as *C. imicola* (25 individuals) in horse farms of Niague and Pout from pond edge and puddle edge, respectively (Tables 2 and 3). *Culicoides kingi* emerged almost exclusively in horse farms of Mbao and Niague from lake edge; only a single individual emerged from puddle edge in horse farm of Pout. The density of emerged species varied according to larval habitats (Figs. 3 and 4).

The overall sex ratio was unbalanced towards females (SR = 0.58), which was also observed for the two most abundant species (*C. oxystoma* and *C. similis*) and all of the positive larval habitats (Table 3). At horse farms of Mbao and Niague, the most productive larval habitats were pond edges (88 and 83 % of the emerging adults, respectively) (Fig. 3, Table 2). At horse farm of Pout, 99 % of the emerging adults (both sexes) were obtained from puddle edge (Table 2). The highest number ($n = 644$) of emerging individuals was found at horse farm of Mbao, followed by horse farm of Pout ($n = 469$) and horse farm of Niague ($n = 307$) (Table 3). The study of emergence

Table 2 Mean (minimum-maximum) number of *Culicoides* individuals emerged from substrate samples per species, site and larval habitat type

Species/Site	Pond edge			Lake edge			Puddle edge			Total number
	A[a]	B[b]	C[a]	A[c]	B[a]	C	A	B	C[d]	
C. oxystoma	49.8 (26–75)	26.3 (3–78)	1.3	10.8 (5–23)	1.3	–	–	–	56.5 (16–160)	568
C. similis	48.5 (6–99)	29.5 (3–54)	–	–	1.3	–	–	–	11.3 (9–20)	362
C. nivosus	37.8 (11–65)	1.5	–	1.5 (1–5)	3.5	–	–	–	41 (3–108)	341
C. kingi	–	–	–	10.3 (3–38)	6.3	–	–	–	0.3	67
C. enderleini	6.5 (1–22)	2.3 (1–8)	–	0.5	0.5	–	–	–	2.3 (1–8)	45
C. imicola	–	3 (1–7)	–	–	–	–	–	–	3.3 (2–5)	25
C. pycnostictus	–	–	–	–	–	–	–	–	1.3	5
C. moreli	–	1	–	–	–	–	–	–	–	4
C. leucosticus	–	0.3	–	–	–	–	–	–	0.3	2
C. expectator	–	0.3	–	–	–	–	–	–	–	1

Abbreviations: *A* horse farm of Mbao; *B* horse farm of Niague, *C* horse farm of Pout
[a]One sampling site sampled
[b]Four to six sampling sites sampled
[c]One to two sampling sites sampled
[d]Four to five sampling sites sampled

dynamics showed marked variations between sites, larval habitats and species (Fig. 5), probably due to the different environmental and meteorological conditions.

Discussion

Larval habitats of *Culicoides* vector species are investigated in many areas in the world where the species occur. In the Afrotropical region where AHSV is endemic, larval habitats of *Culicoides* are almost unknown or require an update except for the southern part of the continent where *Culicoides* larval habitats are much investigated [14, 16, 19].

To our knowledge, this study is the first to identify larval habitats of several *Culicoides* species, including the species potentially involved in AHSV transmission, in the Niayes area, Senegal, West Africa, in horse-related ecosystems. Positive larval habitats were recorded only outside horse farms. No species-specific habitats were identified although *C. kingi* was particularly abundant in lake edge habitats in horse farms of Mbao and Niague; just one individual emerged from puddle edge in horse farm of Pout. Since the last outbreak of AHSV in 2007 in Senegal, several studies have been conducted in the same horse farms in the Niayes area to characterize *Culicoides* species diversity [21, 41, 42], trophic behaviour [42, 49–51] and population dynamics [21, 22] at the adult stage. These surveys conducted on adult populations in these three horse farms using suction black-light traps

Table 3 Sex ratio (SR) of *Culicoides* individuals emerged from substrate samples per species, horse farm and larval habitat type

	Pond edge			Lake edge			Puddle edge			
Species/Sites	A	B	C	A	B	C	A	B	C	Total
C. oxystoma	63 ♂/136 ♀	38 ♂/67 ♀	2 ♂/3 ♀	9 ♂/19 ♀	5 ♀	–	–	–	100 ♂/126 ♀	212 ♂/356 ♀
C. similis	75 ♂/119 ♀	52 ♂/66 ♀	–	–	5♀	–	–	–	17 ♂/28 ♀	144 ♂/218 ♀
C. nivosus	36 ♂/115 ♀	4 ♂/2 ♀	–	1 ♂/5 ♀	5 ♂/9 ♀	–	–	–	72 ♂/92 ♀	118 ♂/223 ♀
C. kingi	–	–	–	21 ♂/20 ♀	4 ♂/21 ♀	–	–	–	1 ♂	26 ♂/41 ♀
C. enderleini	3 ♂/20 ♀	2 ♂/7 ♀	–	2 ♀	1 ♂ /1 ♀	–	–	–	2 ♂/7 ♀	8 ♂/37 ♀
C. imicola	–	2 ♂/10 ♀	–	–	–	–	–	–	4 ♂/9 ♀	6 ♂/19 ♀
C. pycnostictus	–	–	–	–	–	–	–	–	2 ♂/3 ♀	2 ♂/ 3 ♀
C. moreli	–	4 ♀	–	–	–	–	–	–	–	4 ♀
C. leucosticus	–	1 ♂	–	–	–	–	–	–	1 ♂	2 ♂
C. expectator	–	1 ♂	–	–	–	–	–	–	–	1 ♂
Total	177 ♂/390 ♀	100 ♂/156 ♀	2 ♂/3 ♀	31 ♂/46 ♀	10 ♂/41 ♀	–	–	–	199 ♂/265 ♀	519 ♂/901 ♀ (SR = 0.58)

Abbreviations: *A* horse farm of Mbao; *B* horse farm of Niague; *C* horse farm of Pout

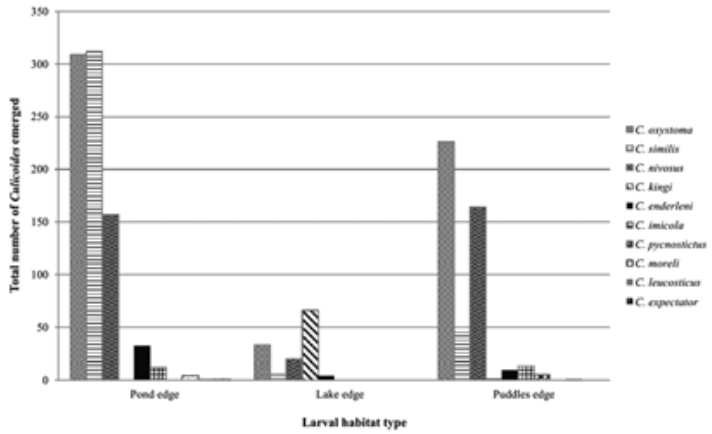

Fig. 3 Number of emerged individuals per species for each larval habitat type

revealed a high species diversity with at least 18 species collected [21, 42], whereas 10 species emerged from the collected substrates in our study. Of these 10 species, eight are proven or suspected biological vectors of viruses of interest in animal health: *Culicoides imicola* major vector of AHSV in Afrotropical region [52–55], *C. oxystoma* [56, 57], *C. kingi*, *C. enderleini*, *C. nivosus*, *C. leucostictus*, *C. pycnostictus* and *C. exspectator* [55]. *Culicoides kingi* is involved in the transmission of *Onchocerca gutturosa*, a parasite of cattle [58].

It is known that the abundance of *Culicoides* larvae collected or emerged from substrates is lower than that of *Culicoides* adults collected using suction black-light traps. Thus, abundance of *C. sonorensis* adults collected in two dairy farms in Northern California using suction black-light traps was weakly correlated with that of the larvae [23]. Auriault et al. [59] followed during three years *C. grahamii* populations at both adult and larval stages in Gabon. These authors did not identify the larval habitats of this species using emergence traps in an environment of debris and decomposing banana trunks while *C. grahamii* adult populations were abundant and caused a nuisance to humans [59].

Interestingly, only semi-aquatic freshwater and saltwater habitats were positive for *Culicoides* larvae in our study; these were similar to some described larval habitats in South Africa [14, 18] and Kenya [15]. Other larval habitats mentioned in the Afrotropical literature such as the dung pats of large herbivores [16, 17] were found negative for *Culicoides* larvae in our study. Indeed, no larvae were found in horse and cattle dungs investigated in our study and all larval habitats identified were located outside the horse farms, in the immediate surroundings, and all represented permanent semi-aquatic habitats.

The absence of positive larval habitats inside horse farms may be due to the daily mechanical disturbance of horse litter. This may affect and limit the attraction of gravid females to these putative breeding sites and oviposition site choice. Indeed, mechanical disturbance may affect larval development of biting midges [60]. Moreover, insecticides are regularly sprayed in some horse boxes against arthropod vectors which could affect

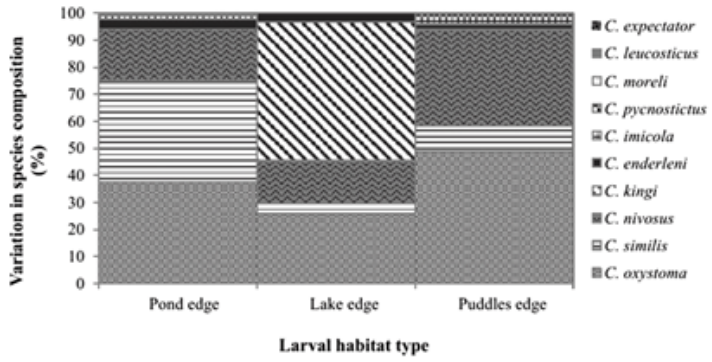

Fig. 4 Species composition for each larval habitat type

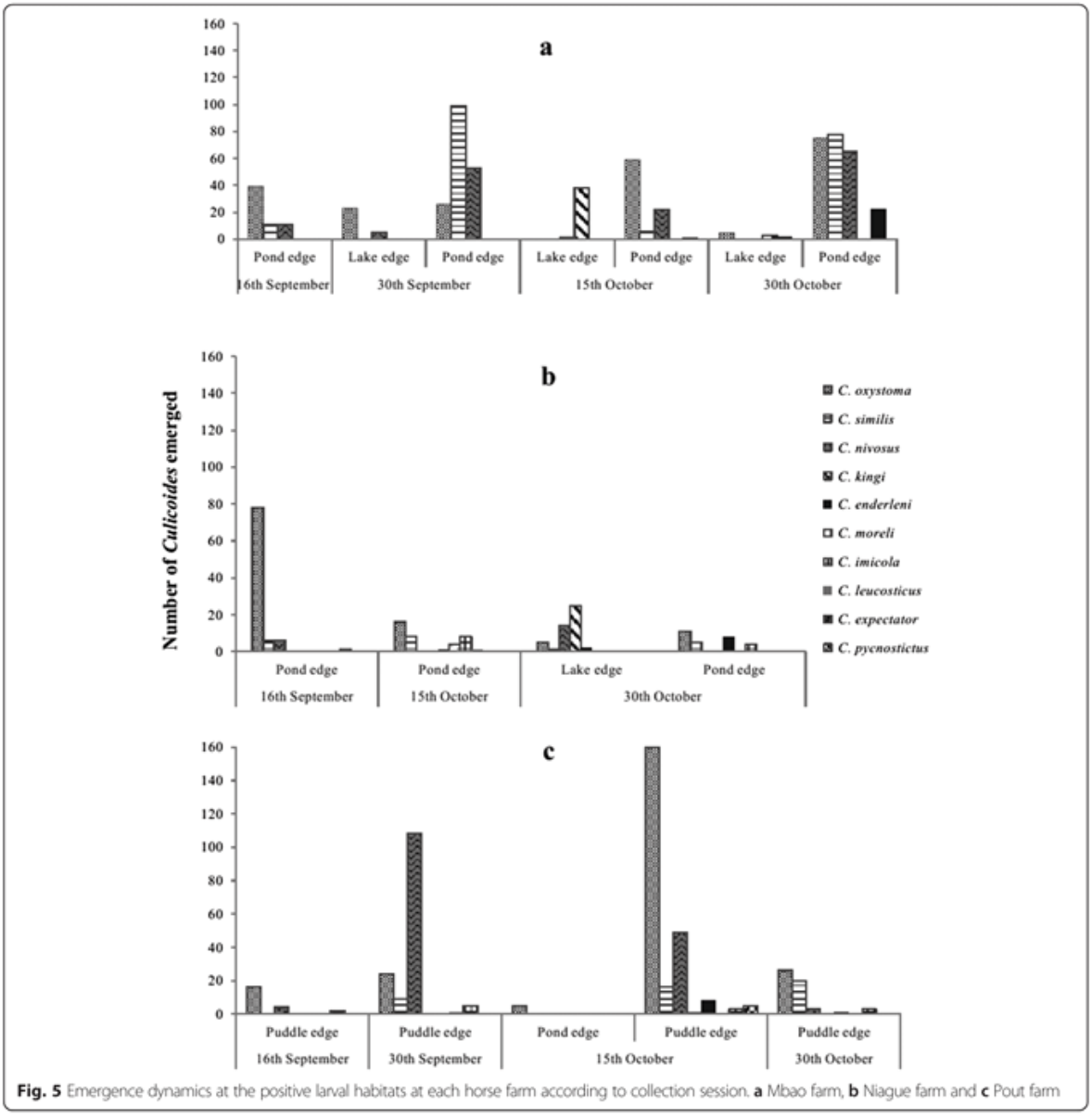

Fig. 5 Emergence dynamics at the positive larval habitats at each horse farm according to collection session. **a** Mbao farm, **b** Niague farm and **c** Pout farm

adult *Culicoides* oviposition and survival. Several studies have examined insecticides against *Culicoides* spp. and depending on the specific product; their efficacy to reduce attack rates and the survival of *Culicoides* spp. was more or less satisfying [60–62].

In our study, the overall sex ratio was unbalanced towards females, which is also observed for other studies [14, 32]. The numbers of emerged individuals and the composition of *Culicoides* spp. varied according to larval habitats and horse farms but also collection sessions (16th September, 30th September, 15th October and 30th October). *Culicoides kingi* and *C. imicola*, two important species of veterinary importance, emerged in very low numbers from field-collected substrate samples whereas adult specimens were abundantly collected at the same period in the same sites using suction black-light traps [21, 22]. The same was observed for *C. imicola* in South Africa by [14, 18]. Different hypothesis could explain these discrepancies. The developmental success and emergence rates might have been impacted in emerging pots in the laboratory. Even though larvae were reared in substrates from their natural habitats, the transfer of samples into the pot, and then from the field to the lab, changes the environmental conditions

and may have induced mortality. Moreover, favourable larval habitats of *C. imicola* may have been probably poorly sampled or not sampled during the field investigations.

Culicoides kingi and *C. oxystoma* were the dominant species found around the brackish lake edges in Mbao and Niague. Further analyses of the substrate physico-chemical properties may provide a better understanding of specific environmental requirements for these species. *Culicoides oxystoma* was found in all positive larval habitats (pond edge, lake edge and puddle edge). Larvae of this species were also found in several aquatic and semi-aquatic habitats in Japan and India, such as paddy fields, stream edges and pond margins [28–30]. *Culicoides oxystoma* has a wide range of larval habitats such as the case of *C. imicola* in several areas where this species is present [14, 16, 18, 24].

Conclusions

The combination of flotation and emergence methods was used to investigate 12 putative larval habitats; of these, three larval habitats were found outside the horse farms, in the immediate surroundings and represented permanent semi-aquatic habitats. No larvae were retrieved from larval habitat types associated with dung (horse dungs, fresh and heap cattle dungs). Although preliminary, these baseline results are very important to further insights into the larval habitats of *Culicoides* spp. in the Niayes area, an AHS endemic region in Senegal, before conducting further studies.

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Availability of data and materials
The data supporting the conclusions of this article are included within the article.

Authors' contributions
AGF and GG designed and supervised the study. MTB, CKB and GG performed the collections sampling and global management of the entomological material. MTB, MF and CKB performed species identification. MTB, AGF, MTS, TB, JB, CG and GG wrote the first draft of the manuscript. All authors revised and approved the final version of the manuscript.

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

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

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Physicochemical characteristics and *Culicoides* diversity of larval habitats in the Niayes area of Senegal

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Abstract

Some biting midges species of the genus *Culicoides* (Diptera: Ceratopogonidae) are the biological vectors of important arboviruses of livestock worldwide, including African horse sickness virus (AHSV). Information on the suitable and productive larval habitats for the main *Culicoides* species is of great importance to further evaluate effective, selective and respectful of the environment control measures and for improving local scale models of vector abundance. In the current study, we combine *Culicoides* larvae collection using flotation method, emergence of adult *Culicoides* from substrate samples and physiochemical measurements of larval habitats to investigate larval development of *Culicoides* with veterinary interest, namely proven or potential vector species of AHSV in the Niayes area of Senegal. A total of 4,716 *Culicoides* larvae and 11, 268 emerging adult *Culicoides* (5,200 ♂ / 6,068 ♀) belonging to 12 species emerged from the substrate samples were collected. The most abundant of *Culicoides* species were distributed as follows: *C. oxystoma* (n = 7346), *C. nivosus* (n = 2134), *C. similis* (n = 706), *C. imicola* (n = 419), *C. distinctipennis* (n = 294), *C. enderleini* (n = 210) and *C. kingi* (n = 102). The presence of *Culicoides* larvae and emerging adult *Culicoides* was strongly associated with pH, salinity and organic matter levels. Abundance of some *Culicoides* species such as *C. imicola*, *C. kingi* and *C. oxystoma* contributes to increasing the risk of transmission AHSV in the Niayes area of Senegal.

Key words. *Culicoides*, African horse sickness virus, larval habitat development, Niayes area

Introduction

Biting midges of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) are involved in the transmission of pathogens (viruses and nematodes) in the world (Mellor *et al.*, 2000; Mullen, 2009; Simonsen *et al.*, 2011). They are the biological vectors of important virus of livestock, including African horse sickness virus (AHSV), Bluetongue virus (BTV), Epizootic hemorrhagic disease virus (EHDV) and Schmallenberg virus (SBV) (Purse *et al.*, 2015). In the Afrotropical region, the AHSV is endemic (Mellor & Hamblin, 2004; Carpenter *et al.*, 2017), and was first recognized in South Africa (Meiswinkel *et al.*, 2004), transmitted by *Culicoides imicola* Kieffer and *C. bolitinos* Meiswinkel (Du Toit, 1944; Venter *et al.*, 2000; Paweska *et al.*, 2003). Recent outbreaks of AHS occurred between 2006 and 2008 in Namibia (Scacchia *et al.*, 2009) and in 2007 in Senegal (Diouf *et al.*, 2012) leading this last country to 1,169 dead horses and considerable economic losses, estimated to 1.4 million euros (Akakpo *et al.*, 2011). In Senegal, 53 *Culicoides* species are recorded including these two important species of veterinary interest *C. imicola* and *C. bolitinos* (Diarra *et al.*, 2014; Fall *et al.*, 2015a), and other species are suspected to be vectors of AHSV such as *C. oxystoma* (Diarra *et al.*, 2015; Fall *et al.*, 2015b; Bakhoum *et al.*, 2016b) but up to date, their formal implication as biological vector has not been identified.

To protect livestock, several methods of prevention and control could be used to limit the host-vector contact by reducing the populations of *Culicoides* species; for a review of techniques to control biting midges, see Carpenter *et al.* (2008), and for an assessment of mermithid nematodes as biological control agents, see Mullens *et al.* (2008). These methods mainly focused on adult *Culicoides* whereas control measures against larvae are not used according to the paucity of knowledge on the larval habitats of *Culicoides* species of veterinary interest (Carpenter *et al.*, 2008). Indeed, successful control of the immature stages of *Culicoides* depends on the identification of their breeding sites, spatial distribution, abundance and ecological factors affecting the choice of larval habitats. Although the larval ecology of some *Culicoides* species of the Palearctic region seems to be well known (Uslu & Dik, 2007; Zimmer *et al.*, 2008; Foxi & Delrio, 2010; Harrup *et al.*, 2013; Zimmer *et al.*, 2014), studies and fundamental information's stay scarce for some species of the Afrotropical region and specifically, in West Africa such as Senegal. Especially, immature life development of *Culicoides* are poorly known, such as species breeding sites preferences, ecological factors involved in this choice or emergence dynamics. Generally, females lay eggs in a rich organic environment of semi-aquatic or wet substrates and larval development

happens preferentially in the uppermost layer of these habitats (Uslu & Dik, 2006). The larvae eat organic matter and microorganisms such as algae, nematodes, bacteria and protozoa or small invertebrates such as nematodes or insects larvae (Blanton *et al.*, 1979; Mullen & Hribar, 1988). Habitats of *Culicoides* immature stages could be very different such as freshwater marshes and swamps, shallow margins of ponds, streams and rivers, peat lands, beaches, around leaking irrigation pipes and water troughs, tree holes and other natural cavities in rotting wood, waterlogged pastures, animal manure, rotting fallen fruits, highly alkaline or saline inland pools and animal dung (Nevill, 1967; Braverman *et al.*, 1974; Dipeolu & Ogunrinade, 1976; Lubega & Khamala, 1976; Ray & Choudhury, 1988; Meiswinkel, 1989; Poddar *et al.*, 1992; Blackwell *et al.*, 1994; Foxi & Delrio, 2010; Jenkins & Young, 2010; Gonzalez *et al.*, 2013; Harrup *et al.*, 2013; Bakhoum *et al.*, 2016a; Labuschagne, 2016) but many factors still remain unknown about the ecology of the immature stages. Some species have a strong adaptive ability and could be find in a huge range of habitats whereas others are poorly adaptive and need specifics requirement such as coprophilic or fruitiphilic species (Nevill, 1967; Zimmer *et al.*, 2014). Although poorly documented, some physicochemical parameters have been found to be important for larval development such as pH (Blackwell *et al.*, 1994; Blackwell *et al.*, 1999; Uslu & Dik, 2010; Harrup *et al.*, 2013), organic matter and mineral (Schmidtmann *et al.*, 2000; Uslu & Dik, 2010).

Knowledge of substrates suitable for *Culicoides* larval development is important, particularly for the main vector species of veterinary interest. This is an important prerequisite to vector control implementation targeting *Culicoides* larval breeding sites. In Senegal, *Culicoides* larval habitats belonging to 10 species have been recently described. *Culicoides* larvae and emerging adults were found at the edges of ponds, puddles and lakes (Bakhoum *et al.*, 2016a). This study was the first one to identify *Culicoides* larval habitats in this country severely impacted by AHS but several factors still remain unknown such as the chemical characteristics which are suitable for larval development of the main vector species, their dynamics and distribution in these habitats. Therefore, in order to characterize *Culicoides* larval habitats and better understand the ecological determinants of larval habitat segregation between *Culicoides* species, we undertook a longitudinal entomological survey in four horse farms in the Niayes area of Senegal (West Africa) were larval habitats have been previously identified (Bakhoum *et al.*, 2016a). The main objectives of this study were: (a) to characterize the physicochemical conditions of *Culicoides* larval habitats, (b) to describe the spatial and

temporal dynamics of *Culicoides* larvae and emerging adult *Culicoides* and c) to describe the emerging adult *Culicoides* diversity.

Materials and Methods

Study area

This study was conducted in four horse farms in the southern part of the Niayes area. The Niayes area of Senegal is a 25- to 30-km wide coastal band stretching over 180 km from Dakar to the southern tip of the Senegal River Delta. The climate is oceanic, with relatively constant humid winds and low thermal amplitudes (Sagna, 2000). In this area, there are two main seasons: the rainy season (July to October) and the dry season, sub-divided into the cold dry season (November to February) and the hot dry season (March to June); vegetation is diversified and mainly composed of steppe and shrub savanna. Rainfall in the Niayes rarely exceeds 350 mm/year (Faye *et al.*, 1995). Animal production is an important activity with: 80 000 to 90 000 cattle, 179 000 sheep and goats, 17 100 equids, and more 5 220 000 poultry (Fall *et al.*, 2000; Bouyer *et al.*, 2014).

Culicoides sampling was conducted in four sites in the Niayes area: Parc de Hann, Mbao, Niague and Pout (Fig. 1) and among these, fourteen larval habitats were monitored 2 times per month from January to December 2015. These larval habitats are distributed as follows (Fig. 1): Two larval habitats of “freshwater lake edge” in Parc de Hann (Ph1 and Ph2), three in Mbao (Mb1 of “pond edge”, and Mb2 and Mb3 of “ saltwater lake edge”), five in Niague (Ng1 of “ saltwater lake edge”, and Ng2, Ng3, Ng4 and Ng5 of “pond edge”), and four larval habitats of “puddle edge” in Pout (Pt1, Pt2, Pt3 and Pt4) were studied.

Fig. 1. Map of geographic location of the fourteen investigated larval habitats (**red dots**). Horse farms in the Niayes area of Senegal are represented as follows: (a) "Parc de Hann" lat 14.728702° and long -17.431764°. (b) "Mbao" lat 14.740963° and long -17.324806°. (c) "Niague" lat 14.826407° and long -17.249563°. (d) "Pout" lat 14.766218° and long -17.036015°. **Google Earth**. April 4, 2017.

Larval habitat sampling

Larval habitats were sampled using a sampling cylinder of 8 cm diameter plug into the ground at 6 centimeters deep. For each larval habitat, four samples were withdrawn. The first one was used to investigate midge larvae in the lab using a direct flotation technique in saturated sugar solution (850 g/liter). *Culicoides* larvae were collected, counted and then preserved in 70° ethanol. The three other substrate samples were used for laboratory emergence and adult collection. Ground samples were placed individually in 200 ml plastic pots covered with a net before being transported to the laboratory (insectarium) to monitor adult emergence. Emerging pots were maintained for 35 days at a temperature of 25 ± 1°C, 80± 10% relative humidity and a light dark photoperiod of 12:12h to allow the retrieval of emerging adult *Culicoides*. The surface of the substrate was sprayed every two days with demineralized water

to prevent desiccation. Each day, emerging adults were collected using a mouth-operated aspirator and then preserved in 70° ethanol. *Culicoides* species identifications were done on emerging adult *Culicoides* using a stereomicroscope (10-40x) and reference identification keys (Boorman, 1989; Glick, 1990; Cornet & Brunhes, 1994; Labuschagne, 2016).

Physicochemical measures

During each field trip, ground sample was collected for physicochemical measures. Six physicochemical parameters were recorded such as carbon (%), conductivity (ms/cm), dry matter (%), organic matter (%), pH and salinity (%) by the Chemistry Laboratory of *Institut Sénégalais de Recherches Agricoles, Laboratoire National de l'Elevage et de Recherches Vétérinaires* (ISRA-LNERV), BP 2057, Dakar, Sénégal.

Data analysis

We performed partial triadic analysis (PTA) (Thioulouse & Chessel, 1987; Thioulouse *et al.*, 2004) to compare the physicochemical parameter assemblage variation among larval habitats and determine the temporal stability of this structure. PTA is a multivariate method that analyzes matrices in a three dimensional data tables, such as a matrix (larval habitat x physicochemical parameters) with a third dimension representing time (months). It is based on the logic of Principal Component Analysis (PCA). Partial triadic analysis is designed to study simultaneously several sub-matrices of quantitative data and to detect within the structure any pattern common to these different sub-matrices in order to extract a multivariate structure that is expressed through the different times (months). The analysis consists of three successive steps called interstructure, compromise and trajectories. The interstructure provides the individual contribution of physicochemical parameter matrices of each month, to the common ecological structure through time and gives a weight to each matrix according to their importance. The compromise calculates an average matrix (compromise table); with a weighed mean of the different physicochemical parameter matrices. The compromise table offers a structure which is then analyzed via PCA to reveal the common structure between the observations for an inferential approach to validating the compromise (Lazraq *et al.*, 2008). The trajectories project each matrix onto the compromise to analyze communalities and discrepancies. The PTA was conducted using the ade4 package (Dray & Dufour, 2007).

Generalized Linear Mixed-Effects Models (GLMM) (McCullagh & Nelder, 1989) were used to assess relationships between physicochemical parameters and the occurrence of *Culicoides*

larvae and emerging adult from larval habitat groups. The models were fitted by maximum likelihood (Laplace Approximation). A Poisson regression mixed-effect model with random effect at the larval habitat groups (G1, G2 and G3) and month of sampling levels, 2/3 of the sample were used for the training dataset and the remaining 1/3 of the sample for the test dataset. The full model contained the untransformed continuous physiochemical parameters carbon, conductivity, dry matter, organic matter, pH and salinity; and larval habitat groups. Final models were obtained using a backwards-stepwise selection-based procedure, such that variables that did not contribute significantly to explain variation in *Culicoides* larvae or emergence of *Culicoides* were successively eliminated on the basis of the Akaike Information Criterion (AIC) (Akaike, 1973). The root mean square error (RMSE) was used for validation.

The densities of *Culicoides* larvae and emerging adult *Culicoides* per month and per larval habitat groups were plotted. A log10 (n + 1) transformation was applied to density data per month. *Culicoides* densities (log10 (n + 1)) were compared between larval habitat groups, and then by month by performing a Kruskal-Wallis test (Hollander & Wolfe, 1973). The apparent density (*AD*) was estimated for *Culicoides* larvae and emerging adult *Culicoides* as the number of specimens (sp) collected per larval habitat group (h) and per month (m). To determine the species composition in each larval habitat, species accumulation curves were plotted. The diversity of communities was assessed using the Simpson index (*H*) calculated with the "*diversity*" command of the *vegan* package. Species richness (*S*) was determined as the number of *Culicoides* species collected. The dominant species index (*d*) was estimated using the Berger-Parker equation: $d = Ni/N$, where *Ni* is the number of individuals of the i^{th} species and *N* the total number of sampled individuals (all species). This index ranges from 0 to 1, and d values close to 1 indicate high dominance.

All statistical analyses were performed using R v3.3.2 (https://www.r-project.org/).

Results

Direct flotation and Culicoides emerged

During the one-year sampling period (2 times per month from January to December 2015), direct flotation carried out on substrate samples from fourteen larval habitats from four horse farms in the Niayes area of Senegal allowed the collection of 74, 716 *Culicoides* larvae. In addition, 11 268 adult *Culicoides* (5,200 ♂ / 6,068 ♀) belonging to 12 species emerged from the substrate samples keep in the laboratory (Table 1). *Culicoides* species were distributed as

follows: *C. oxystoma* (n = 7346), *C. nivosus* (n = 2134), *C. similis* (n = 706), *C. imicola* (n = 419), C. *distinctipennis* (n = 294), *C. enderleini* (n = 210), *C. kingi* (n = 102), *C. pycnostictus* (n = 24), *C. leucostictus* (n = 21), *C. gambiae* (n = 7), *C. exspectator* (n = 3) and *C. austeni* (n = 2).

Table 1. Composition and density of *Culicoides* species in each larval habitat group. The index d value of dominant species is marked in bold.

	Number of specimen				Dominance index d		
	G1	G2	G3	Total	G1	G2	G3
C. austeni	0	2	0	2♀	0.000	0.000	0.000
C. distinctipennis	216	77	1	130♂ / 164♀	**0.651**	0.007	0.002
C. enderleini	1	204	5	27♂ / 183♀	0.003	0.019	0.011
C. exspectator	2	1	0	1♂ / 2♀	0.006	0.000	0.000
C. gambiae	1	6	0	1♂ / 6♀	0.003	0.001	0.000
C. imicola	2	415	2	197♂ / 222♀	0.006	0.039	0.004
C. kingi	1	30	71	29♂ / 73♀	0.003	0.003	**0.154**
C. leucostictus	2	19	0	14♂ / 7♀	0.006	0.002	0.000
C. nivosus	14	1,855	265	931♂ / 1,203♀	0.042	**0.177**	**0.574**
C. oxystoma	8	7,252	86	3,547♂ / 3,799♀	0.024	**0.692**	**0.186**
C. pycnostictus	4	20	0	11♂ / 13♀	0.012	0.002	0.000
C. similis	81	593	32	312♂ / 394♀	**0.244**	0.057	0.069

Abbreviations: G1 = larval habitat group of freshwater lake type; G2 = larval habitat group of pond and puddle types; G3 = larval habitat group of saline lake type

Partial triadic analysis

A PTA was run in order to visualize the seasonal variations of physicochemical parameters during the study (Fig.2). As shown by the interstructure, which represents the physicochemical parameters structure over the years (Fig. 2a), the parameters were globally stable along the year. The first two axes of the Principal Component Analysis of the compromise table (Fig. 2b, c) present a clear separation between three groups of larval habitats and explain 91% of total inertia. The first group 1 (G1) corresponds to two larval habitats that are freshwater lake edges in the Parc de Hann. This habitat is characterized by a high content of organic matter (mean = 8.70%) and carbon (mean = 5.03%); and an acidic pH (mean = 6.05)]. The second group (G2) is composed of 9 larval habitats distributed between two sites (Pout and Niague) and all-corresponds to ponds and puddles edges. These two-habitat types share physicochemical similarities characterized by high value of dry matter

(mean = 71.17%), neutral pH (mean = 7.25) and very low salinity). The third group corresponds to three larval habitats of saline lake type distributed between two sites (Mbao and Niague) [with high water electrical conductivity (mean = 15.06 ms/cm) and salinity (mean = 1.52%); and a basic pH (mean = 8.14)]. The table weights were similar, ranging from 0.26 to 0.31 (Fig. 2d; Table 2). In addition, the cos2 values revealed that the compromise table was representative of the individual matrices, ranging from 0.77 to 0.90 (Fig. 2d; Table 2). Therefore, these values indicated that physicochemical parameter assemblages varied moderately among months.

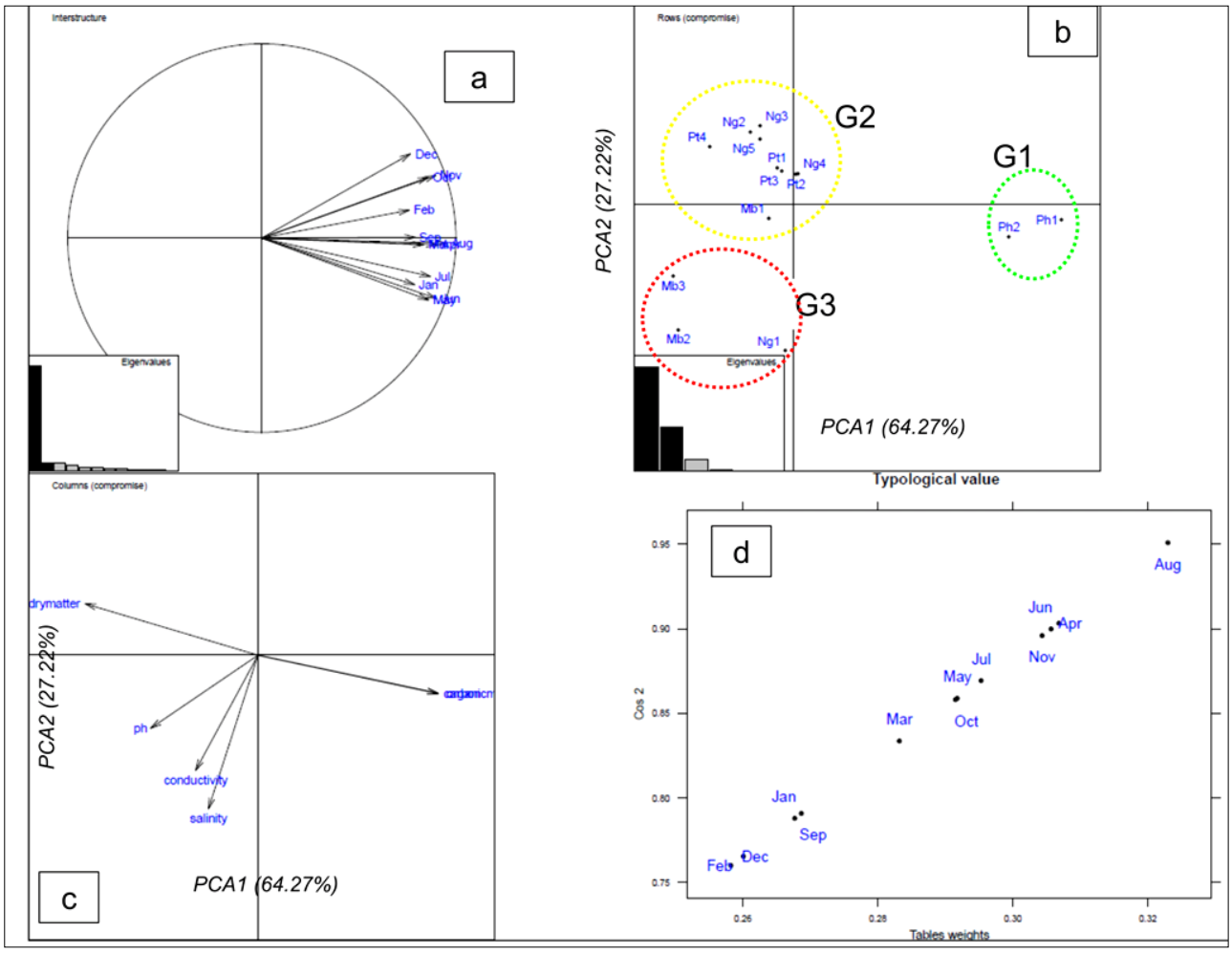

Fig. 2. Interstructure month ordination from vector correlation coefficients (a), ordination plots of the first two axes (*PCA1 = 64.27%* and *PCA2 = 27.22%*) of the Principal Component Analysis of the compromise table (b and c), and table weights and cos2 values for each month represented in a scatter plot (d).

Table 2: Vectorial correlation (RV) coefficients of the individual physicochemical parameter matrices of each month, and their table weights and cos2 values

	Vectorial correlation coefficients													
													cos2 values	Table weights
Months	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec		
Jan	1.00												0.79	0.27
Feb	0.58	1.00											0.76	0.26
Mar	0.61	0.71	1.00										0.83	0.28
Apr	0.62	0.74	0.80	1.00									0.90	0.31
May	0.73	0.53	0.68	0.77	1.00								0.86	0.29
Jun	0.78	0.60	0.70	0.78	0.84	1.00							0.90	0.31
Jul	0.57	0.63	0.75	0.82	0.76	0.85	1.00						0.87	0.30
Aug	0.78	0.70	0.74	0.85	0.82	0.85	0.82	1.00					0.95	0.32
Sep	0.58	0.44	0.54	0.66	0.69	0.72	0.66	0.73	1.00				0.79	0.27
Oct	0.60	0.63	0.70	0.73	0.64	0.70	0.66	0.80	0.70	1.00			0.86	0.29
Nov	0.63	0.68	0.71	0.77	0.66	0.73	0.72	0.85	0.72	0.87	1.00		0.90	0.30
Dec	0.56	0.54	0.54	0.64	0.60	0.57	0.57	0.72	0.61	0.70	0.78	1.00	0.77	0.26

Factors driving presence-absence of Culicoides

Physicochemical parameter values (i.e. carbon, conductivity, dry matter, organic matter, pH and salinity) varied moderately among months (Fig. 2; Table 2). Therefore, we used average values across the period as predictors in the GLM models of presence. Among the six variables initially considered as predictors of *Culicoides* larval habitats, the stepwise selection retained pH, salinity and organic matter as the variable combinations that best discriminated both the presence of *Culicoides* larvae and emerging adult *Culicoides* Moreover, regression coefficients of these three predictor variables are significant (p-values <0.05; Table 3).

Table 3: Generalized Linear Mixed Models for *Culicoides* larvae and emerging adult *Culicoides* from fourteen larval habitats placed in tree groups.

Explicative variables	**Regression coefficients**	**p-value**
***Culicoides* larvae**		
(Intercept)	-3.07306	0.0245
pH	0.31491	< 2e-16
Salinity	0.40969	< 2e-16
Organic matter	0.54518	< 2e-16
Emerged *Culicoides*		
(Intercept)	-1.946848	0.191
pH	0.337442	< 2e-16
Organic matter	0.658630	< 2e-16
Salinity	-0.956603	< 2e-16

Relative abundance and diversity of Culicoides in larval habitat group

The highest apparent densities (*AD*) of emerging adult *Culicoides* from the substrate samples and *Culicoides* larvae collected by floatation were observed in G2 with values of 97 and 40.86 respectively (Table 4), highlighting that puddles and ponds are the most productive habitats. Also, the *Culicoides* diversity was highest in G2 with a specific richness (*S*) value of 12 and a Simpson index (*D*) of 0.48, followed by G1 (*S*= 11, *D*= 0.51) and G3 (*S*= 7, *D*= 0.60) (Table 4). The species composition and dominance index (*d*) revealed that in G1 (*i.e.* larval habitat group of freshwater lake type), *C. distinctipennis* was the dominant species with a dominance index value of 0.651 followed by *C. similis* (*d*= 0.244) (Table 1). In G2 (*i.e.* larval habitat group of pond and puddle types), *C. oxystoma* and *C. nivosus* were predominant with dominance index values of 0.692 and 0.177 respectively (Table 1). Both species were again the most abundant in G3 (*i.e.* larval habitat group of saline lake type), but with highest index for *C. nivosus* (*d*= 0.574) followed by *C. oxystoma* (*d*= 0.186) and *C. kingi* (*d*= 0.154) (Table 1). Although these species were dominant in their respective habitats, we should note that among the 12 species collected, 7 were found in three habitat groups (C. *distinctipennis*, *C. enderleini, C. imicola*, *C. kingi*, *C. nivosus*, *C. oxystoma* and *C. similis*), 4 only in G1 and G2 freshwater habitats (*C. exspectator*, *C. gambiae*, *C. leucosticus* and *C. pycnostictus*) and *C. austeni* only found in G2 (Table 1).

Table 4. Apparent density (sp/h/m) and diversity of *Culicoides* in different larval habitat groups

	AD of Larvae	**AD of *Culicoides***	**S**	**Simpson index (D)**
G1	4	13.83	11	0.51
G2	40.86	97	12	0.48
G3	5.75	12.83	7	0.60

Abbreviations: sp = specimen; h = larval habitat group; m = month; AD = apparent density; S = Species richness; G1 = larval habitat group of freshwater lake type; G2 = larval habitat group of pond and puddle types; G3 = larval habitat group of saline lake type

Analysis of temporal and spatial dynamics of *Culicoides* larvae and emerging adult *Culicoides* revealed slight variations (Fig. 3). Temporal variation for *Culicoides* larvae was significant ($\chi 2$= 35.373, df= 11, *p-value*< 0.001) along the year, but not for emerging adult *Culicoides* ($\chi 2$= 18.548, df= 11, *p-value*= 0.069). The highest densities of *Culicoides* larvae were observed between February and August with peaks of dominance in April and May (Fig. 3a) whereas it was less marked for emerging adults (Fig. 3c). Spatial variations for *Culicoides* larvae and emerging adult *Culicoides* (Fig. 3b, d) were not significant ($\chi 2$= 26.277, *p-value*=

0.994 and $\chi 2$= 64.681, *p-value*= 0.772 respectively) and showed both a similar pattern of spatial distribution among larval habitat groups.

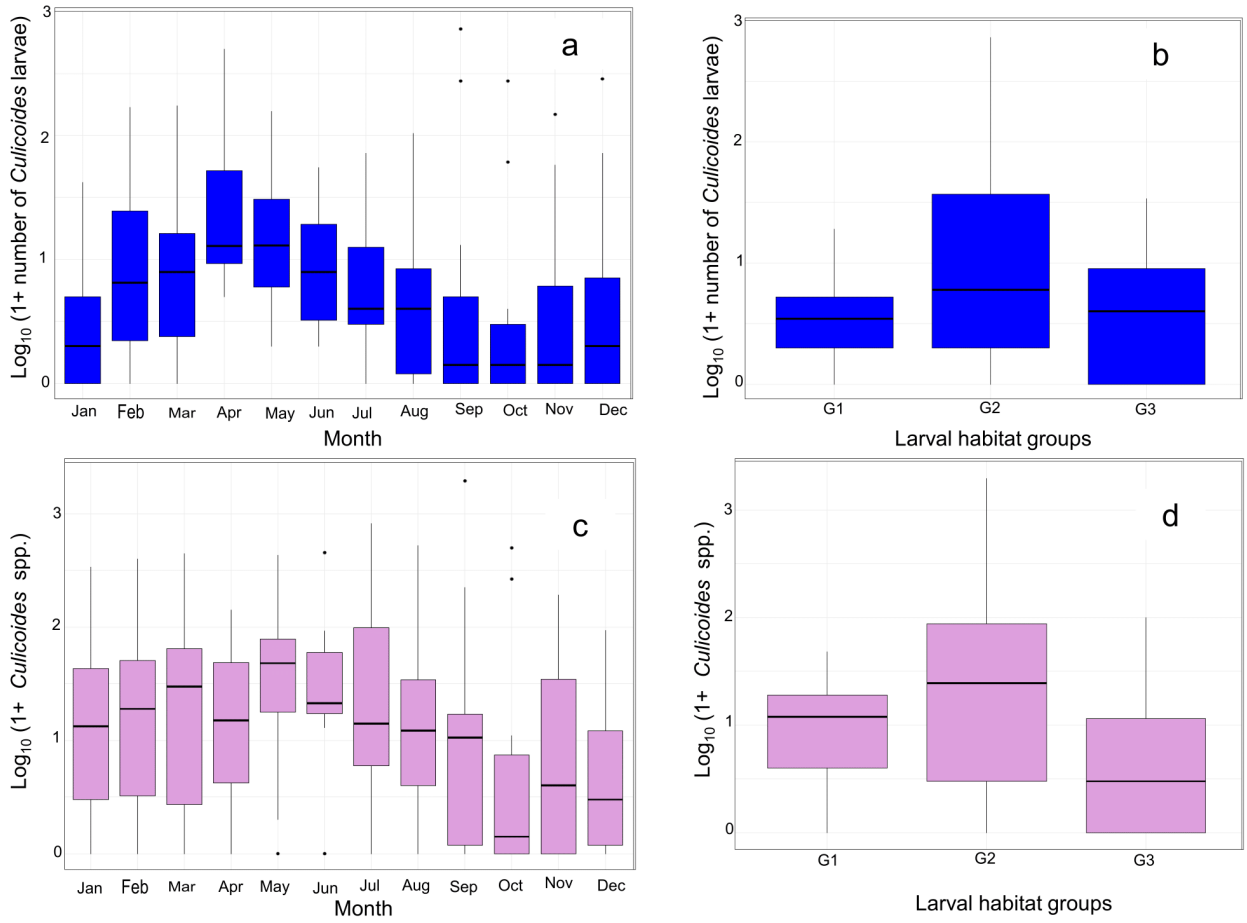

Fig. 3. Spatial and temporal variations of the density of *Culicoides* larvae (a, b) and emerging adult *Culicoides* (c, d).

Discussion

Larval ecology of *Culicoides* species remains one of the most overlooked aspects of their life cycle. Although it is well known that species develop in a wide range of wet substrates (Zimmer *et al.*, 2014), several aspects such as species-species requirements, distribution and associations are not known, especially for Afrotropical species. In a previous study, we identify for the first time larval habitats of several *Culicoides* species in the Niayes area, Senegal, West Africa. Here, we performed a longitudinal entomological survey of three *Culicoides* larval habitat types and described the physicochemical characteristics of these habitats and species composition, the dynamics and diversity of *Culicoides* species found in it.

The larval habitats of *Culicoides* known in this area were edges of lakes (fresh and saltwater), ponds and puddles (Bakhoum *et al.*, 2016a). Partial Triadic Analysis performed on the 14 larval habitats highlighted three clusters according to their physicochemical similarities. The group G1 represents freshwater larval habitats of the Parc de Hann. This habitat is characterized by high value of organic matter, carbon and acidic pH. The Parc de Hann contains a freshwater lake surrounded by a dense forest that produces important amount of organic materials through leaf and wood decomposition. This decomposition of organic materials probably leads to an acidic pH that characterizes this habitat and may select for species that could develop in such conditions. Dominant species found in this habitat were *Culicoides distinctipennis* and *C. similis*. Both species are probably specific to forested area in association to high organic matter. In the literature, *C. distinctipennis* has been already collected in Senegal and mainly found in forested area such as the Bandia and Ndioum-Kalo forest (Cornet, 1969). However, this is a widespread species in Africa (Cornet, 1969; Glick, 1990; Labuschagne, 2016) and it's ecological preferences for larval habitats seems linked to the amount of organic matter as it was shown in Salisbury area in Rhodesia, where *C. distinctipennis* was dominant along drainage canals in a muddy intermediate organic matter environment(Braverman, 1978). The group G2 clustered all ponds and puddles edges habitats. Although these habitats are distributed in three different sites, Mbao, Niague and Pout (Fig. 1 and 2), they share the same physicochemical properties. These two-habitat types (ponds and puddles edges) present high values of dry matter that may reflects the ground composition in Mbao, Niague and Pout that is mainly mineral, composed of sand. *Culicoides oxystoma* was the most abundant species in this habitat type and has been found rarely in G1 and G3 (0.001% and 0.01% of the specimens collected) that probably reflect its ecological preferences. Therefore, this species seems to breed preferentially in sandy freshwater habitats but no information's were found in the literature on the breeding preferences of this species. This observation is in accordance to previous studies conducted in Mbao, Niague and Pout where *C. oxystoma* was the main abundant species in adult collection (Diarra *et al.*, 2014; Diarra *et al.*, 2015; Fall *et al.*, 2015a). The group G3 is also composed of larval habitats distributed between two sites, Mbao and Niague and corresponds to saltwater lake edges. There are characterized by a strong salinity, conductivity and basic pH. Indeed, the two habitats in Mbao are on the edges of two lagoons that are connected to the sea and under tide influences. These lagoons are located in an urbanized area and are very polluted by wastewater. The saltwater lake of Niague or "Lac Rose" was also connected to the sea but seems preserved with no visual pollution. The three dominant species found in this habitat

were *C. kingi*, *C. nivosus* and *C. oxystoma*. The first species has been described in the literature as a species that breeds preferentially in salt mud (Cornet, 1969) but it seem to be not exclusive and could be also found in freshwater habitats. Indeed, in Kenya, this species has been only retrieved in salty marshes (Lubega & Khamala, 1976) but in our study, 30% and 69% of the specimens collected emerging from G2 and G3 habitats respectively.

Species, which were found in a small numbers, are not necessarily rare. In a number of instances, it is possible that only their marginal breeding sites were sampled and their main breeding site remains undiscovered.

In 2015, Bakhoum et al. (2016) found ten *Culicoides* species emerged from these three habitat types. Here, in this second study three news species were found in these larval habitats: *C. austeni*, *C. distinctipennis* and *C gambiae*, and one species were absent *C. moreli*. Moreover, although these three habitat groups present different physicochemical properties, seven species have been found in all of them (C. *distinctipennis*, *C. enderleini, C. imicola*, *C. kingi*, *C. nivosus*, *C. oxystoma* and *C. similis*). This result highlight that most of the species collected are not specific to one larval habitat and could develop in a range of larval habitats and physicochemical conditions ranging from acid to basic pH and fresh to salt water. It contrasts with the larval bio-ecology of Palearctic species those are generally species-specific and found in one or few habitat types (Zimmer *et al.*, 2014). The ability of these species to colonize fresh and salt water reveals important information about their physiology. Indeed, in order to survive in salty conditions, insect's larvae must regulate their water balance (i.e. osmoregulation) and are confined to water whose osmotic concentration is less than larval haemolymph (Bradley, 1987). Interestingly, no species specific of brackish larval habitats have been found suggesting that ability to thrive in these habitats is probably a consequence of ecological diversification of freshwater species to tolerance to saltwater environment. It is also important to note that the three species of veterinary interest, namely, *C. oxystoma*, *C. imicola* and *C. kingi*, all belong to this group. Therefore, this ability to develop in a wide range of larval habitat could be one of the main constraints to the implementation of vector control operation targeting larval breeding sites. On the opposite, four species seems to be confined to freshwater larval habitats (*C. exspectator*, *C. gambiae*, *C. leucostictus* and *C. pycnostictus*) and only *C. austeni* was found in one larval habitat type (puddles edges).

Results obtain from our models; pH, salinity and organic matter are the best factors to describe presence-absence of *Culicoides* larvae and productive habitats (i.e. producing adults).

Therefore, according to our results, *C. distinctipennis* and *C. similis* were found to be dominant species in habitats of freshwater lake edge that have high organic matter, carbon and an acidic pH. Our results indicate the dominance of species of *C. kingi*, *C. nivosus* and *C. oxystoma* in habitats of saline lake edge which have high salinity and a basic pH; but also *C. nivosus* and *C. oxystoma* were dominant species in habitats of pond and puddle edges which have high dry matter and a neutral pH. In United Kingdom, *C. impunctatus* was strongly associated with substrate moisture level and peat-based mires of acidic pH (5.0–6.5) (Blackwell *et al.*, 1994; Blackwell *et al.*, 1999). Harrup et al. (2013) found that the presence of emerging adult *Culicoides* and specifically *C. obsoletus* in larval habitats was strongly associated with substrate moisture level and a neutral or alkaline pH. It has been previously reported that *C. imicola* favors conditions that are semi-moist (Nevill, 1967; Foxi & Delrio, 2010). This appears is confirmed by the fact that almost all adults of *C. imicola* emerged from pond and puddle edges, less moist than lake edges, which have high dry matter and a neutral pH. The organic and dry matters are essential for larval development. It was reported that larval development is optimum in the habitat rich in nutrients (Blanton *et al.*, 1979; Mullen & Hribar, 1988; Zimmer *et al.*, 2014).

In the current study, temporal variation for *Culicoides* larvae was significant and the highest density values were observed between February and August with peaks of dominance in April and May in the studied larval habitats. Although marginally significant (p=0.069), *Culicoide*s emergence from substrate collection also shows a similar pattern.

We conclude that the difference in the density and assemblage of *Culicoides* observed in the three larval habitat groups is the consequence of differences of these larval habitats according to physicochemical parameters. Our results highlight that the presence of some *Culicoides* species such as *C. imicola*, *C. kingi* and *C. oxystoma* contributes to increasing the risk of transmission AHSV in the Niayes area of Senegal. The emergence of AHS in Senegal started in the Dakar region particularly in the Niayes with two outbreaks in March and June 2007 (Diouf *et al.*, 2012). This period coincides with the peaks of abundance of *Culicoides* larvae. In this regard, egg laying for *Culicoides* larvae development was preceded by feeding of *Culicoides* females on vertebrate hosts namely horses in the Niayes area of Senegal. However, larval habitats around the horse farms particularly puddle and pond edges could play a significant role in the re-emergence of the African horse sickness virus in the Niayes area of Senegal. Thus selective and respectful of the environment control measures (application of larvicides, destruction of larval habitats…) should be quite effective and,

furthermore, should reduce the density of emerging adult *Culicoides* in larval habitats, namely potential or proven vector species of AHSV in the Niayes area of Senegal.

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Article 5: **Bakhoum M.T.**, Sarr M., Fall A. G., Huber K., Labuschagne K., Gardès L., Fall M., Seck M.T., Gimonneau G., Bouyer J., Baldet T., Garros C. (***In prep***.) DNA barcoding for *Culicoides* biting midges (Diptera: Ceratopogonidae) and application for *Culicoides* larvae identification in the Niayes area of Senegal, West Africa

DNA barcoding for *Culicoides* biting midges (Diptera: Ceratopogonidae) and application for *Culicoides* larvae identification in the Niayes area of Senegal, West Africa

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Abstract

The ecological and veterinary importance of *Culicoides* need for rapid and reliable identification of these biting midges. We assessed the efficiency of DNA barcoding for species identification of *Culicoides*. A total of 229 cytochrome c oxidase subunit 1 sequences were obtained from 40 adult *Culicoides* species in Afrotropical region. DNA barcodes provided 97% correct identification.

We also assessed the utility of these DNA barcodes to discriminate among *Culicoides* larvae collected in the Niayes area of Senegal. A total of 935 COI sequences of *Culicoides* larvae were successfully identified corresponding at 8 *Culicoides* species. Of these species, *Culicoides oxystoma* had the highest percentage (66.42%) followed by *C. nivosus* (21.06%). DNA barcode trees were largely congruent with phylogenies based on previous molecular and morphological analyses, but revealed inconsistencies.

Key words: *Culicoides*, cytochrome oxidase I, DNA barcoding, Afrotropical region, Senegal

Introduction

Biting midges species of the genus *Culicoides* (Diptera: Ceratopogonidae) are the biological vectors of important arboviruses of livestock worldwide, such as African horse sickness virus (AHSV), Bluetongue virus (BTV), Epizootic hemorrhagic disease virus (EHDV) and Schmallenberg virus (SBV) (Purse *et al.* 2015). African horse sickness virus is a lethal arbovirus of equids that is biologically transmitted between equids by competent vectors of the *Culicoides* genus (Carpenter *et al.* 2017). African horse sickness is recorded in Africa and Arabic peninsula and is ranked as among the most lethal of viral infections known in horses with rates of fatality in naive horse populations can reach 80-90% (Carpenter *et al.* 2017). Massive AHS epizootic outbreaks occurred in Senegal in 2007 (Akakpo *et al.* 2011; Diouf *et al.* 2012).

Accurate vector species identification is critical for understanding disease epidemiology as well as *Culicoides* species vector bio-ecology to implement selective and respectful control measures. *Culicoides* species diversity in the Afrotropical region reaches 190 described species (Bakhoum *et al.* 2013; Cornet & Chateau 1970; Cornet *et al.* 1974; Glick 1990; Itoua *et al.* 1987; Labuschagne 2016; Meiswinkel & Dyce 1989). The last revision of the fauna of

Senegal revealed 41 of 53 recorded *Culicoides* species with 19 species collected in the horse-baited trap in which *C. oxystoma*; *C. imicola* and *C. kingi* were largely dominant (Bakhoum *et al.* 2013; Fall *et al.* 2015). These *Culicoides* species are placed in 9 subgenera (*Avaritia*, *Beltranmyia*, *Culicoides*, *Meijerehelea*, *Monoculicoides*, *Pontoculicoides*, *Remmia*, *Synhelea*, and *Trithecoides*), 9 species groups unplaced to subgenus (Accraensis, Albovenosus, Bedfordi, Dekeyseri, Inornatipennis, Milnei, Neavei, Nigripennis, and Similis) and miscellaneous species, not placed in any group representing 28% of Afrotropical fauna.

Morphological taxonomy of *Culicoides* biting midges species of are challenging because of their small size, limited resources for adult identification and virtually no identification keys for the immature stages. .

DNA barcoding was proposed as a solution to the limitations of traditional taxonomy using DNA sequence similarity (Hebert *et al.* 2003). DNA barcode for species identification used a small portion (≈ 658 bp) of the mitochondrial gene cytochrome *c* oxidase unit I (COI) to assign specimen sequence to a voucher species library (Hebert *et al.* 2003). Successful DNA barcoding depends on the distinction between intraspecific and interspecific genetic divergence, which could be influenced by the time since speciation and effective population size. Indeed, the performance of DNA barcoding can vary within the same group of specimens among geographic regions and ecosystems (Elias *et al.* 2007). Species with large effective population sizes can have high intraspecific genetic diversity, which could overlap with interspecific divergence. Interspecific variation might not yet be realized on a generation of species sharing a recent common ancestor, the species could be genetically paraphyletic or polyphyletic (Avise 2000). And also, imperfect taxonomy also could lead to erroneous identifications (Meyer & Paulay 2005).

In our study, we examined the efficiency of DNA barcoding to differentiate 41 *Culicoides* species collected in different sites in the Afrotropical region. We assessed the phylogenetic relationships of these DNA barcodes to provide species identifications in agreement with morphology-based taxonomic identifications. We present also an assessment of the utility of DNA barcoding to discriminate among *Culicoides* larvae in the Niayes area of Senegal, West Africa.

Materials and Methods

Culicoides collection

Adult *Culicoides* specimens were collected in different sites localized in 12 countries of the Afrotropical region through different field missions between 2009 and 2016. *Culicoides* were collected using Onderstepoort black light trap or CDC trap set up at the vicinity of farms or equids (Fig. 1). Morphological identification was carried out under a binocular microscope using the available identification keys for the Afrotropical region (Boorman 1989; Cornet & Brunhes 1994; Glick 1990; Labuschagne 2016). For each specimen, wings and genitalia were dissected prior to DNA extraction processing and slide-mounted to record the morphological features. All samples are kept for further works at Cirad, UMR117 ASTRE, Montpellier, France and are available upon request to the corresponding authors.

Culicoides larvae sampling was conducted in four sites in the Niayes area: Parc de Hann, Mbao, Niague and Pout (Fig. 1) and among these, fourteen larval habitats were monitored 2 times per month from January to December 2015.

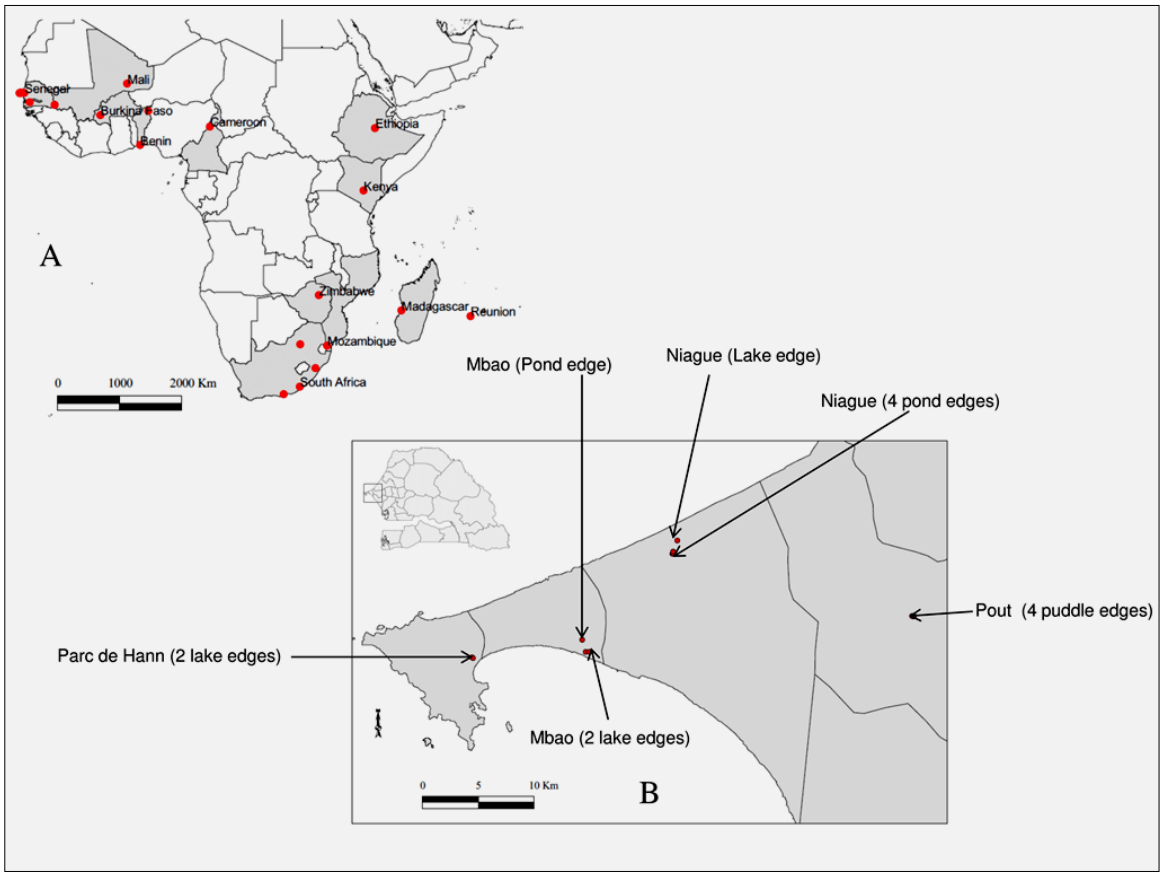

Fig. 1: Collection sites of 40 *Culicoides* species in Afrotropical region (A) and *Culicoides* larvae in the Niayes area of Senegal, West Africa (B). Details of sampling sites are given in Table S1 (additional details).

DNA extraction, polymerase chain reaction and sequencing

Genomic DNA was individually extracted using the NucleoSpin® Tissue DNA Kit (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's instructions and maintained at –20°C until further use. PCR amplification reactions were performed in a 25 µL total reaction volume containing 1X of Qiagen buffer, 1 mM of $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of forward primer LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATATTG G-3′, 0.2 µM of reverse primer HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al. 1994), 1.25 U of Qiagen Polymerase Taq and 0.4 ng/µL of genomic DNA. The PCR cycling conditions were as follows: an initial denaturation step at 94 °C for 5 min followed by 5 cycles of 94°C for 30 s, 45°C for 40 s, 72°C for 1 min, 35 cycles of 94 °C for 30 s, 51°C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Positive and negative controls for the amplification reactions were carried out at every PCR round. The PCR products were separated on 1.5% agarose gels and the products were sequenced using the same primers as used in PCR amplifications (https://www.genewiz.com). All sequences were deposited in GenBank (see Table S1 for additional details).

Data analysis

The DNA sequences were edited in Geneious R6 (Biomatters, http://www.geneious.com/). DNA sequences were aligned using MACSE (Multiple Alignment of Coding SEquences accounting for frame shifts and stop codons) (Ranwez *et al.* 2011). Test of substitution saturation (Xia *et al.* 2003) was performed in DAMBE (Xia & Xie 2001). Statistics and tests of barcoding efficacy were performed using *spider* package (Brown *et al.* 2012) implemented in R v3.3.2 (https://www.r-project.org/). Tests of barcoding efficacy including "*nearest neighbour*" (CulNN), "*threshold analyses*", "*best close match*" and "*monophyly of each species*" (CulMono) are not identification tools, but they permit to investigate whether sequences can be used for species identification (Brown *et al.* 2012; Meier *et al.* 2006). By default, the function *threshID* mimics the "*species identification*" method used by BOLD (Barcode of life database) and offers a threshold based criterion of 1%. It is often a good rule, but it may not always be appropriate to every dataset (Meyer & Paulay 2005). However we investigated the number of true positive, false negative, false positive and true negative identifications at a given threshold, plus the cumulative error (false negative + false positive) by creating a range of threshold values 0.1% to 10%, testing these values and plotting the result. Using *localMinima* function of the *spider* package (Brown *et al.* 2012), we calculated a threshold appropriated at our dataset of *Culicoides* species

identification. Interspecific and intraspecific sequence divergences based on the Kimura-2 parameter were calculated using MEGA v6 (Tamura *et al.* 2013). Haplotype and nucleotide diversity were calculated using DnaSP Version 5 (Rozas *et al.* 2003).

Phylogenetic relationships of species were estimated using Bayesian Inference (BI) and Maximum Likelihood (ML) under a substitution model found using jModelTest (Darriba *et al.* 2012). The BIC implemented within jModelTest was used to determine the most suitable evolutionary model(s). The BI analyses were performed using MrBayes 3.2.3 (Ronquist *et al.* 2012). Two simultaneous and independent runs consisting of sixteen Metropolis-coupled Markov chain Monte Carlo (MCMC) running 50 million generations were used, with a tree sampling every 1,000 generations to calculate posterior probabilities (PP). In order to investigate the convergence of the runs, we investigated the split frequencies and Effective Sample Size (ESS) of all the parameters, and plotted the log-likelihood of the samples against the number of generations in Tracer 1.5 (http://BEAST.bio.ed.ac.uk/Tracer). A value of ESS>1096 was found as a good indicator of convergence. The ML analyses were conducted with the best model selected using PhyML 3.0 (Guindon S. *et al.* 2010). We also assessed the barcode tree profiles, using neighbour-joining (NJ) method implemented in MEGA 5 (Tamura et al. 2011). Branch support for NJ and ML was calculated using the bootstrapping method with 1,000 replicates.

DNA barcode dataset of 231 COI sequences from 41 *Culicoides* species (see Table 1 and Table S1 for additional details) was transformed as a blast database using *makeblastdb* of the Blast software v2.2.31 (Zhang *et al.* 2000). To discriminate *Culicoides* species within the larvae generated sequences, data including 958 COI sequences from *Culicoides* larvae was used as query in BLAST search in DNA barcode data transformed.

Results

PCR products and DNA sequences from samples

PCR was successful for 229 samples from 40 species of adult *Culicoides* collected in different sites localized in 12 countries of the Afrotropical region. Of 4,716 larvae collected in fourteen larval habitats in the Niayes area of Senegal; 1632 (stage L1-L2: 773; stage L3-L4: 859) were selected for molecular identification, according to their abundance and stage in each larval habitat. PCR amplifications failed for 99 out of 773 L1-L2 stage samples. In contrast, all selected samples of stage L3-L4 were successfully amplified (859). All PCR

products were successfully sequenced. And all sequences were deposited in BOLD.
Cytochrome Oxidase Subunit 1 sequences from *C. candolfii* from Gabon [accession numbers: KC986403 and KC986404] (Delecolle *et al.* 2013) were used in this study.

DNA Barcode dataset and sequence diversity

For DNA barcode dataset, a total of 229 sequences from 40 adult *Culicoides* species (see Table S1 for additional details) and 2 sequences from *C. candolfii* from Gabon [accession numbers: KC986403 and KC986404] (Delecolle *et al.* 2013) is considered (Table 1): *C. bolitinos*, *C. candolfii*, *C. grahamii*, *C. gulbenkiani*, *C. imicola*, *C. kanagai*, *C. kibatiensis*, *C. kwagga*, *C. loxodontis*, *C. miombo*, *C. pseudopallidipennis*, *C. sp.* #20, *C. sp.* #22, *C. sp.* #54 dark form, *C. tororoensis* and *C. tuttifrutti* (*Avaritia* subgenus), *C. nivosus* (*Beltranmyia* subgenus), *C. brucei* and C. *magnus* (*Culicoides* subgenus), *C. distinctipennis*, *C. leucostictus* and *C. pycnostictus* (*Meijerehelea* subgenus), *C. engubandei* (*Pontoculicoides* subgenus), *C. enderleini*, *C. kingi*, *C. nevilli*, *C. oxystoma*, *C. schultzei* and *C. subschultzei* (*Remmia* subgenus), *C. ravus*, *C. similis* and *C. tropicalis* (*Synhelea* subgenus), *C. austeni*, *C. isioloensis*, *C. milnei*, *C. moreli* and *C. zuluensis* (Milnei group), *C. neavei* and *C. ovalis* (Neavei group), and *C. macintoshi* and *C. murphyi* (unplaced species) (Table S1, Supplementary information). DNA barcode sequences shows an alignment of 663 pb, with an average nucleotide composition of A = 28.1%, T = 40.7%, C = 15.9% and G = 15.4%.
Intraspecific nucleotide diversity was calculated for all species and ranged from 0.00 to 0.107 (Table 1). High values were reported in *C. bolitinos* (mean: 0.066; range: 0.059-0.074), *C. similis* (mean: 0.073; range: 0.065-0.08); and *C. neavei* (mean: 0.107; range: 0.093-0.122). Haplotype diversity for each species ranged from 0 (i.e. all specimens had identical sequences) to 1 (i.e. all specimens had unique sequences) (Table 1).
The sequences obtained in this study showed high interspecific divergence values with a mean K2P of 0.226 (range: 0.192-0.3) (Table 1).

Table 1. Haplotype characteristics and levels of intra and interspecific diversity of DNA barcode dataset

Taxon	n	n_{hap}	Intra	H	π	Inter
C. austeni	1	1	0	0	0	0.239-0.359 (0.3)
C. bolitinos	13	11	0.059-0.074 (0.066)	0.974 ± 0.039	0.05666 ± 0.00605	0.095-0.292 (0.205)
C. brucei	3	3	0.021-0.031 (0.026)	1 ± 0.272	0.0244 ± 0.00747	0.156-0.322 (0.216)
C. candolfii	2	1	0	0	0	0.194-0.303 (0.249)
C. distinctipennis	7	7	0.031-0.041 (0.036)	1 ± 0.076	0.03138 ± 0.006	0.14-0.339 (0.225)
C. enderleini	22	22	0.046-0.054 (0.05)	1 ± 0.014	0.03952 ± 0.01284	0.126-0.29 (0.213)
C. engubandei	2	2	0.006-0.015 (0.01)	1 ± 0.5	0.01036 ± 0.00518	0.2-0.301 (0.244)
C. grahamii	3	3	0.046-0.059 (0.052)	1 ± 0.272	0.04717 ± 0.01435	0.239-0.338 (0.277)
C. gulbenkiani	3	3	0.011-0.019 (0.015)	1 ± 0.272	0.01414 ± 0.00462	0.179-0.303 (0.23)
C. imicola	17	17	0.016-0.021 (0.019)	1 ± 0.02	0.01307 ± 0.00227	0.108-0.289 (0.204)
C. isioloensis	2	2	0.002-0.007 (0.005)	1 ± 0.5	0.00465 ± 0.00233	0.202-0.313 (0.261)
C. kanagai	1	1	0	0	0	0.195-0.324 (0.244)
C. kibatiensis	1	1	0	0	0	0.173-0.314 (0.244)
C. kingi	7	7	0.039-0.049 (0.044)	1 ± 0.076	0.03693 ± 0.00957	0.121-0.277 (0.203)
C. kwagga	2	2	0.003-0.009 (0.006)	1 ± 0.5	0.0062 ± 0.0031	0.123-0.308 (0.209)
C. leucostictus	3	3	0.012-0.021 (0.016)	1 ± 0.272	0.01626 ± 0.00514	0.14-0.319 (0.216)
C. loxodontis	2	2	0.002-0.007 (0.005)	1 ± 0.5	0.00469 ± 0.00235	0.132-0.342 (0.228)
C. macintoshi	3	3	0.002-0.006 (0.004)	1 ± 0.272	0.00413 ± 0.00119	0.169-0.292 (0.225)
C. magnus	6	5	0.01-0.017 (0.013)	0.933 ± 0.122	0.01322 ± 0.00344	0.156-0.278 (0.202)
C. milnei	1	1	0	0	0	0.213-0.295 (0.239)
C. miombo	13	9	0.016-0.02 (0.018)	0.872 ± 0.00833	0.01458 ± 0.00752	0.135-0.284 (0.202)
C. moreli	6	6	0.014-0.021 (0.018)	1 ± 0.096	0.0172 ± 0.00496	0.198-0.312 (0.243)
C. murphyi	15	13	0.003-0.006 (0.005)	0.981 ± 0.031	0.00464 ± 0.00056	0.142-0.283 (0.201)
C. neavei	2	2	0.093-0.122 (0.107)	1 ± 0.5	0.09502 ± 0.04751	0.184-0.324 (0.234)
C. nevilli	9	9	0.036-0.045 (0.04)	1 ± 0.052	0.03804 ± 0.0052	0.059-0.297 (0.197)
C. nivosus	5	4	0.032-0.041 (0.037)	0.9 ± 0.161	0.03225 ± 0.01149	0.181-0.335 (0.23)
C. ovalis	1	1	0	0	0	0.18-0.359 (0.26)
C. oxystoma	16	16	0.046-0.055 (0.051)	1 ± 0.022	0.04249 ± 0.01431	0.119-0.302 (0.205)
C. pseudopallidipennis	13	12	0.047-0.058 (0.052)	0.987 ± 0.035	0.04294 ± 0.00824	0.088-0.3 (0.211)
C. pycnostictus	2	2	0.005-0.013 (0.009)	1 ± 0.5	0.00905 ± 0.00452	0.161-0.338 (0.222)
C. ravus	4	4	0.05-0.064 (0.057)	1 ± 0.177	0.05345 ± 0.01652	0.17-0.304 (0.225)
C. schultzei	2	2	0.013-0.024 (0.019)	1 ± 0.5	0.0181 ± 0.00905	0.101-0.279 (0.195)
C. similis	7	5	0.065-0.08 (0.073)	0.857 ± 0.137	0.06208 ± 0.01551	0.172-0.297 (0.222)
C. sp. #20	6	6	0.007-0.011 (0.009)	1 ± 0.096	0.00885 ± 0.00112	0.157-0.314 (0.221)
C. sp. #22	5	5	0.002-0.006 (0.004)	1 ± 0.126	0.00407 ± 0.00093	0.108-0.281 (0.201)
C. sp. #54	1	1	0	0	0	0.208-0.338 (0.275)
C. subschultzei	6	6	0.009-0.015 (0.012)	1 ± 0.096	0.01197 ± 0.00165	0.059-0.229 (0.192)
C. tororoensis	2	2	0.004-0.011 (0.008)	1 ± 0.5	0.00765 ± 0.00382	0.179-0.304 (0.235)
C. tropicalis	3	3	0.009-0.016 (0.013)	1 ± 0.272	0.01252 ± 0.00543	0.149-0.288 (0.207)
C. tuttifrutti	4	4	0.008-0.014 (0.011)	1 ± 0.177	0.00961 ± 0.00415	0.088-0.285 (0.193)
C. zuluensis	8	6	0.036-0.047 (0.042)	0.929 ± 0.084	0.03759 ± 0.00912	0.191-0.324 (0.249)

n, number of COI sequences; n_{hap}, number of COI haplotypes; *Intra*, Range of genetic divergence within taxa (mean); *H*, haplotype diversity values ± standard deviation; *π*, nucleotide diversity values ± standard deviation; *Inter*, Range of genetic divergence between taxa (mean)

Identification Success Rates using DNA Barcode data

The Nearest Neighbour identification of closest *Culicoides* specimen (CulNN) to the target species was 97.83% (226 of 231 sequences) of correct identification (Fig. 2). The function *threshID* according to threshold analysis similar to the method of specimen identification used by BOLD, i.e. 1% (CulBOLD), found 142 of 231 sequences matched with the query (61.47%) (Fig. 2). Indeed, the cumulative error was 36.64% at 1% of threshold value, (i.e. nucleotide divergence is higher than 1%, BOLD threshold value).

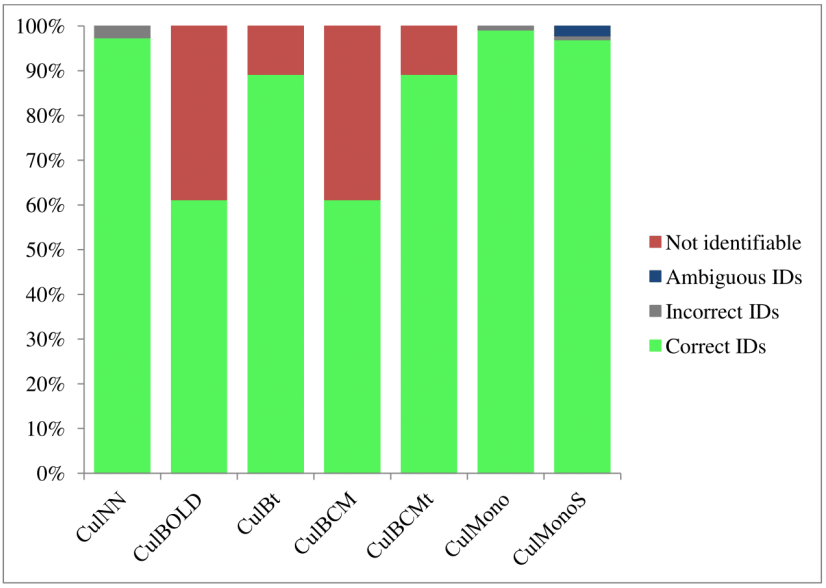

Fig. 2: Barplots of seven measures of identification success. CulNN: Nearest Neighbour; CulBOLD: Threshold analysis (1% threshold); CulBt: Threshold analysis (4.10% threshold); CulBCM: Best close match (1% threshold); CulBCMt: Best close match (4.10% threshold); CulMono: Proportion of monophyly on a NJ tree; CulMonoS: Proportion of monophyly on a NJ tree, singletons returned as unidentified.

False-positive and false-negative error rates were totaled for a range of threshold values (0.1% to 10%), and combined error minimized (Fig. 3). The minimum cumulative error of false positive and false negative identifications showed the optimum threshold of 4.10% (Fig. 3). The function *threshID* () using optimum threshold (CulBt) was found 207 of 231 sequences matched with the query (89.61%) (Fig. 2). The '*best close match*' (CulBCM) showed same results to *threshID* () (Fig. 2). Species monophyly method has showed a large number of specimens (230 of 231 sequences) which are monophyletic over a neighbour-joining (NJ) tree (Fig. 2).

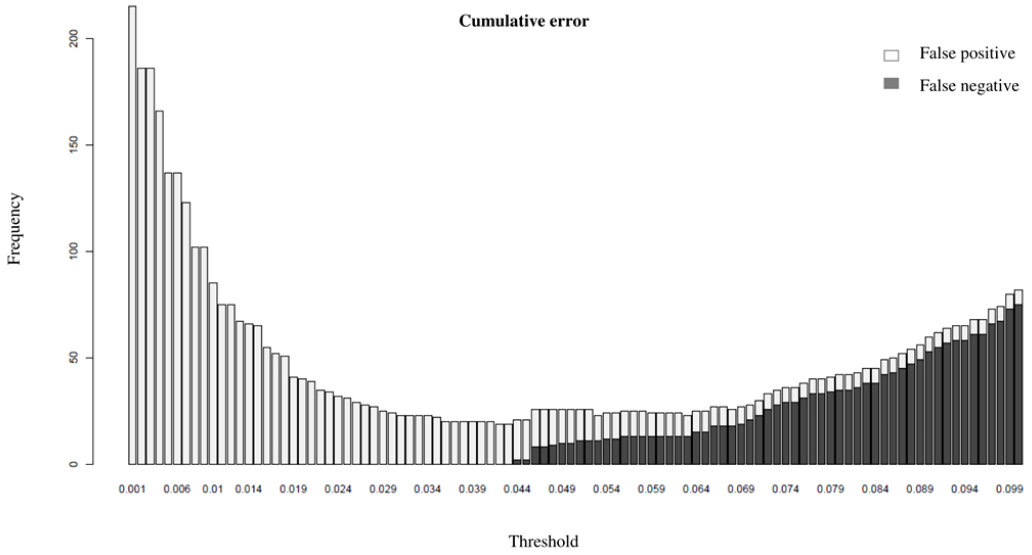

Fig. 3: The optimum threshold showed by the minimum cumulative error of false positive and false negative identifications (4.10% for our DNA barcode data).

Molecular identification of Culicoides larvae

COI sequencing from immature materials were successful for 958 out of 1,632 larvae (58.6%). PCR amplifications failed for 99 out of 773 L1-L2 stage samples. In contrast, all selected samples of stage L3-L4 were successfully amplified (859 samples). The overall rate of COI sequences matched within in DNA barcode reference library used as query in BLAST search was 99.79% (956/958). No matches were found for two sequences. Of 956 COI sequences of *Culicoides* larvae matched within in DNA barcode reference library, 935 were successfully identified corresponding at 8 *Culicoides* species (Table 2). Of these species, *Culicoides oxystoma* had the highest percentage (66.42%), followed by *C. nivosus* (21.06%), *C. distinctipennis* and *C. similis* with a slightly lower percentage at 4% (Table 2).

Table 2. Nucleotide sequence identities between the larvae and sequences in DNA barcode data of *Culicoides* species in the partial sequence of COI

***Culicoides* spp.**	**No. Of matched larvae**	**Range of nucleotide identities (mean) (%)**
C. distinctipennis	34	95-99 (97)
C. enderleini	27	95-99 (97)
C. imicola	1	99
C. kingi	17	99
C. nivosus	197	97-100 (98.5)
C. oxystoma	621	90-100 (95)
C. pycnostictus	1	97
C. similis	37	92-99 (95.5)
Total	935	95.5-99 (97.25)

Trees using and COI sequence variation

All three analysis methods (BI, ML and NJ) revealed similar tree topologies (Figs. 4 and 5). However, minor differences included the placement of *C. kibatiensis* and *C. grahamii* members of the subgenus *Avaritia* in both NJ and MP trees. *C. moreli* (Milnei group) clustered with members of the Dasyops group (*Avaritia*) in the NJ tree (Fig. 4) but a separate clade in the ML and BI trees (Fig. 5). *Culicoides* species clades represented in the three molecular phylogenies were concordant with morphological identifications. All COI sequence larvae matched within in DNA barcode reference library clustered with corresponding *Culicoides* species.

The ML method has been considered the best approach compared with other methods (Guindon S. *et al.* 2010; Kuhner & Felsenstein 1994); thus, phylogenetic relationships are interpreted using ML tree (Fig. 5). Species of Imicola group (*C. sp.* #22, *C. imicola*, *C. kwagga*, *C. bolitinos*, *C. tuttifrutti*, *C. loxodontis* and *C. pseudopallidipennis*) clustered with *C. tororoensis* and *C. sp*. #20 (PP= 0.91/ BS= 67). However, *C. miombo* (Imicola group) was close to the clade of other members of the subgenus *Avaritia* (*C. gulbenkiani*, *C. kanagai*, *C. sp.* #54 and *C. candolfii*) (PP= 0.75/ BS= 60). *Culicoides grahamii* clustered with *Culicoides* larvae (NG4Q9H, no identified) (PP= 1/ BS= 60). Specimens morphologically identified as belonging to *Culicoides* subgenus (*C. brucei* and *C. magnus*) clustered with *Culicoides* larvae (PH2U12C, no identified) and strongly supported (PP= 1/ BS= 87). Subgenus *Culicoides* clade is close to *C. zuluensis* which clustered with *Culicoides* larvae (NG5T5A, no identified). *Culicoides similis* and *C. exspectator* (*Synhelea*) clustered with *C. engubandei* (*Pontoculicoides*) and also *C. nivosus* (*Beltranmyia*), but not supported. *Culicoides tropicalis* clustered with *Culicoides* larvae (PH1P2F, no identified). The subgenus *Remmia* recovered as monophyletic with support (PP= 1/ BS= 71) and contains six species (*C. enderleini*, *C. kingi*, *C. nevilli*, *C. oxystoma*, *C. subschultzei* and *C. schultzei*). *Culicoides* species formed a clade not supported: *C. ravus* (*Synhelea*), *C. pycnostictus* (*Meijerehelea*), *C. ovalis* (Neavei group), *C. macintoshi* (unplaced species), *C. neavei* (Neavei group), *C. leucostictus* and *C. distinctipennis* (*Meijerehelea*). This clade is close to *C. murphyi* which clustered with 3 *Culicoides* larvae (no identified) (PP= 0.7/ BS= 56). *Culicoides milnei* clustered with *C. austeni* and *C. isioloensis* (Milnei group).

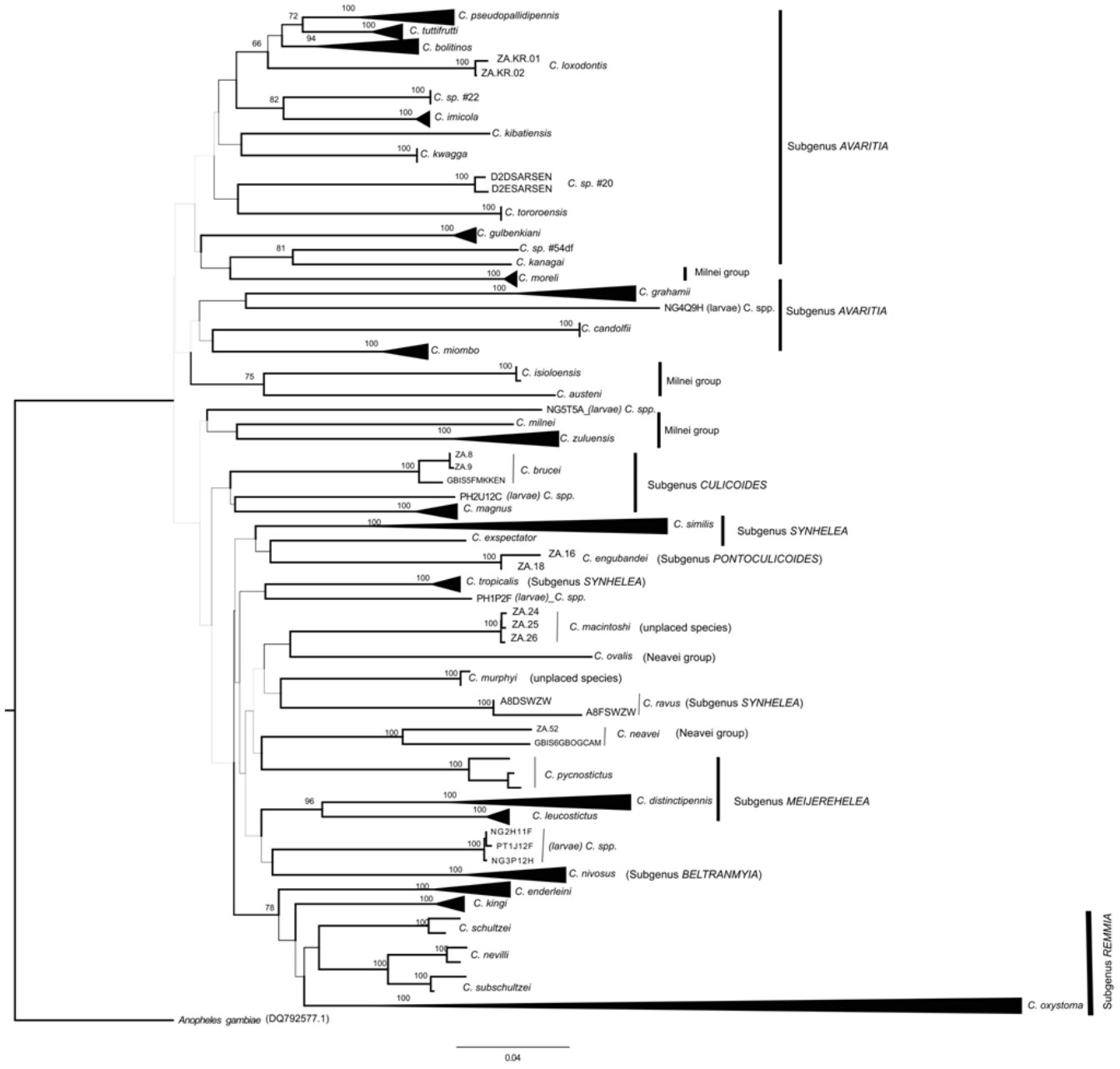

Fig. 4. Neighbour-joining phylogenetic tree based on Kimura two-parameter genetic distances for 215 haplotypes of the mitochondrial cytochrome c oxidase subunit 1 sequences of 42 *Culicoides* species in Afrotropicale region and 935 COI sequences of *Culicoides* larvae from Senegal. Bootstrap values over 50% are displayed over branches.

Fig. 5. Maximum-likelihood phylogenetic tree for 215 haplotypes of the mitochondrial cytochrome c oxidase subunit 1 sequences of 42 *Culicoides* species in Afrotropicale region and 935 COI sequences of *Culicoides* larvae from Senegal. Bootstrap values over 50% and posterior probability are displayed over branches.

Discussion

Insights into the stability or change of the biodiversity require species identification on a broad scale. Biodiversity questions have become a major public issue especially when the species are involved in pathogen transmission. Species identification using DNA sequence similarity was proposed as a solution to the limitations of traditional taxonomy. The utility of DNA sequences for taxonomic or barcoding purpose is based on the nucleotide divergence (Hebert *et al.* 2003; Tautz *et al.* 2003) and need critical assessment before use.

Our study presents the first DNA barcode analysis of the genus *Culicoides* in the Afrotropical region for 41 species. The provision of DNA barcode data for *Culicoides* species, in particular species with medical and veterinary interest, in the Afrotropical region fills an important gap in our knowledge of the phylogeny of these species and identification of immature *Culicoides*. The tests of barcoding efficacy including species monophyly, 'best close match' (Meier *et al.* 2006), threshold analysis and nearest neighbour identification (Austerlitz *et al.* 2009) showed a satisfactory success rate based on proposed criteria. Over the entire dataset, there was slight overlap between intraspecific diversity (mean: 0.053, range: 0.00 - 0.107) and interspecific divergence (mean: 0.226; range: 0.192-0.3). Indeed, for a better molecular delimitation of species, it is important that nucleotide diversity within species is no greater than divergence between species (Meyer & Paulay 2005).

This DNA barcode data was used for identifying a *Culicoides* larvae species. Generally two techniques have been used to identify *Culicoides* larvae based on identification of emerging adults: (i) emergence traps to cover possible larval habitats and allow collecting and identifying adult midges (Dipeolu & Ogunrinade 1976; Harrup *et al.* 2013; Jenkins & Young 2010) and (ii) collection of samples, such as mud or cattle dung, kept in laboratories for several weeks until adult midges emerged and are identified (Bakhoum *et al.* 2016a; Uslu & Dik 2007; Zimmer *et al.* 2008). These methods were advantageous because emerging adult midges could be sorted with well-examined morphological keys. However, they are not suitable for the rapid identification, because of the delayed emergence of adult midges and maintenance efforts necessary to incubate samples until emergence of adult midges in the laboratory. Moreover, these two methods have one main drawback in impacting survival of immature stages. Therefore, using emerging adults to monitor immature dynamics could seriously underestimate species diversity and abundance. In contrast, molecular identification assays using COI sequence similarity were proposed as a solution to limit time consumption and maintenance efforts (Yanase *et al.* 2013). However, we faced low PCR amplification level

with L1-L2 larvae (success of 12.80%). This could be explained by these stages being small and having low concentration of genomic DNA. The comparison of the COI larvae sequences with those of the DNA barcode (see Table S1 for additional details) used as query in BLAST search was a success (99.79%). Taking into account of the optimal threshold of our DNA barcode data (4.10%), we were able to validate the identification of 918 larvae midges for 8 *Culicoides* species: C. *distinctipennis*, *C. enderleini*, *C. imicola*, *C. kingi*, *C. nivosus*, *C. oxystoma*, *C. pycnostictus* and *C. similis*. Only one specimen of *C. imicola* and *C. pycnostictus* were found. In contrast, *C. oxystoma* and *C. nivosus* were largely dominant (66.41% and 21.06% respectively).

Of these 8 species identified, *C. imicola* is regarded as the most important and proven vector species of African horse sickness (Paweska *et al.* 2003; Venter *et al.* 2000) and bluetongue virus (Venter *et al.* 2006); *C. kingi* is involved in the transmission of *Onchocerca gutturosa*, a widespread parasite of Sudanese cattle (El Sinnary & Hussein 1980); and *C. oxystoma* is a well-known vector of bovine arboviruses bovine arboviruses such as Akabane virus (Oem *et al.* 2013; Yanase *et al.* 2005). *Culicoides oxystoma* and *C. kingi* are suspected of being vector of African horse sickness in the Niayes area of Senegal (Bakhoum *et al.* 2016b; Fall *et al.* 2015) based on their abundance and trophic behavior. Larvae of *C. oxystoma* shared several aquatic and semi aquatic habitats, such as pond edge, puddle edge (Bakhoum *et al.* 2016a) and paddy fields (Yanase *et al.* 2013). In contrast, the main larval habitat of *C. kingi* was lake edge (Bakhoum *et al.* 2016a). Although *C. imicola* was sometimes abundant in a suction light trap of the OVI type set up at the vicinity of farms or equids in the Niayes area of Senegal (Diarra *et al.* 2014; Diarra *et al.* 2015), their larval habitats are still to be explored.
Knowledge of larval habitats of *C. kingi* and *C. oxystoma* could facilitate control measures based on immature development stages. DNA barcode data developed herein will be helpful in achieving a better understanding of *Culicoides* larval habitats in the Afrotropical region.

Additional details

Table S1. *Culicoides* specimen details, including COI sequences, associated BOLD ID and sample location information.

ID Specimen	Species	Locality	Province	Country	BOLD ID
A12HPHSEN	*C. austeni*	Parc de Hann	Dakar	Senegal	Submitted
KEN54	*C. bolitinos*	Munukathi	Kitui	Kenya	Submitted
B3BMAMG	*C. bolitinos*	Masoandro	Toamasina	Madagascar	Submitted
B5EMAMZ	*C. bolitinos*	Maputo	Maputo	Mozambique	Submitted
E5EMKKEN	*C. bolitinos*	Munukathi	Kitui	Kenya	Submitted
E5GMKKEN	*C. bolitinos*	Munukathi	Kitui	Kenya	Submitted
E5AMKKEN	*C. bolitinos*	Munukathi	Kitui	Kenya	Submitted
REBG01	*C. bolitinos*	Le Tampon	Saint-Denis	France-Reunion Island	Submitted
REBG02	*C. bolitinos*	Le Tampon	Saint-Denis	France-Reunion Island	Submitted
E4GMKKEN	*C. bolitinos*	Munukathi	Kitui	Kenya	Submitted
KEN55	*C. bolitinos*	Munukathi	Kitui	Kenya	Submitted
ZAKW01	*C. bolitinos*	Kwazulu-Natal	Kwazulu-Natal	South Africa	Submitted
ZAKW02	*C. bolitinos*	Kwazulu-Natal	Kwazulu-Natal	South Africa	Submitted
ZA33	*C. bolitinos*	Port Elizabeth	Eastern Cape	South Africa	Submitted
GBIS5FMKKEN	*C. brucei*	Munukathi	Kitui	Kenya	Submitted
ZA8	*C. brucei*	Nelspruit	Mpumalanga	South Africa	Submitted
ZA9	*C. brucei*	Nelspruit	Mpumalanga	South Africa	Submitted
GBIS1ENGSEN	*C. distinctipennis*	Niague	Dakar	Senegal	Submitted
GBIS1FNGSEN	*C. distinctipennis*	Niague	Dakar	Senegal	Submitted
GBIS1GNGSEN	*C. distinctipennis*	Niague	Dakar	Senegal	Submitted
GBIS1HNGSEN	*C. distinctipennis*	Niague	Dakar	Senegal	Submitted
A10BPHSEN	*C. distinctipennis*	Parc de Hann	Dakar	Senegal	Submitted
A10DPHSEN	*C. distinctipennis*	Parc de Hann	Dakar	Senegal	Submitted
D6EPLGML	*C. distinctipennis*	Gao	Gao	Mali	Submitted
D10GBOGCAM	*C. enderleini*	Bokke Garoua	Garoua	Cameroon	Submitted
B6HMAMZ	*C. enderleini*	Maputo	Maputo	Mozambique	Submitted
C7ASARSEN	*C. enderleini*	Saraya	Kedougou	Senegal	Submitted
D8CPLGML	*C. enderleini*	Gao	Gao	Mali	Submitted
F5FAWPET	*C. enderleini*	Awash Park	Afar	Ethiopia	Submitted
G4DPLGML	*C. enderleini*	Gao	Gao	Mali	Submitted
G4GBOSBEN	*C. enderleini*	Tori-Bossito	Atlantique	Benin	Submitted
G4HBOSBEN	*C. enderleini*	Tori-Bossito	Atlantique	Benin	Submitted
GBIS8EBEN	*C. enderleini*	Ouidah	Atlantique	Benin	Submitted
B6GMAMZ	*C. enderleini*	Maputo	Maputo	Mozambique	Submitted
C5EMBSEN	*C. enderleini*	Mbao	Dakar	Senegal	Submitted
D7HPLGML	*C. enderleini*	Gao	Gao	Mali	Submitted
D8APLGML	*C. enderleini*	Gao	Gao	Mali	Submitted
D8BPLGML	*C. enderleini*	Gao	Gao	Mali	Submitted
E6GAWPET	*C. enderleini*	Awash Park	Afar	Ethiopia	Submitted
F1HMKKEN	*C. enderleini*	Munukathi	Kitui	Kenya	Submitted

(Table S1. Continued)

ID Specimen	Species	Locality	Province	Country	BOLD ID
F2BAWPET	*C. enderleini*	Awash Park	Afar	Ethiopia	Submitted
B1FMAMG	*C. enderleini*	Masoandro	Toamasina	Madagascar	Submitted
B1GMAMG	*C. enderleini*	Masoandro	Toamasina	Madagascar	Submitted
B1HMAMG	*C. enderleini*	Masoandro	Toamasina	Madagascar	Submitted
B6FMAMZ	*C. enderleini*	Maputo	Maputo	Mozambique	Submitted
G5ABEN	*C. enderleini*	Ouidah	Atlantique	Benin	Submitted
ZA18	*C. engubandei*	Amsterdam	Mpumalanga	South Africa	Submitted
ZA16	*C. engubandei*	Amsterdam	Mpumalanga	South Africa	Submitted
REU49	*C. grahamii*	Le Tampon	Saint-Denis	France-Reunion Island	Submitted
REU50	*C. grahamii*	Le Tampon	Saint-Denis	France-Reunion Island	Submitted
REU51	*C. grahamii*	Le Tampon	Saint-Denis	France-Reunion Island	Submitted
E4AMKKEN	*C. gulbenkiani*	Munukathi	Kitui	Kenya	Submitted
GBIS10E	*C. gulbenkiani*	Komga	Eastern Cape	South Africa	Submitted
GBIS11A	*C. gulbenkiani*	Komga	Eastern Cape	South Africa	Submitted
A11HPHSEN	*C. imicola*	Parc de Hann	Dakar	Senegal	Submitted
D4GSALSEN	*C. imicola*	Salikegne	Kolda	Senegal	Submitted
A12GPHSEN	*C. imicola*	Parc de Hann	Dakar	Senegal	Submitted
A12BPHSEN	*C. imicola*	Parc de Hann	Dakar	Senegal	Submitted
B9APOSEN	*C. imicola*	Pout	Thies	Senegal	Submitted
A12DPHSEN	*C. imicola*	Parc de Hann	Dakar	Senegal	Submitted
B5DMAMZ	*C. imicola*	Maputo	Maputo	Mozambique	Submitted
D5BBOGCAM	*C. imicola*	Bokke Garoua	Garoua	Cameroon	Submitted
F2CAWPET	*C. imicola*	Awash Park	Afar	Ethiopia	Submitted
G12EMBSEN	*C. imicola*	Mbao	Dakar	Senegal	Submitted
G12FMBSEN	*C. imicola*	Mbao	Dakar	Senegal	Submitted
GBIS10A	*C. imicola*	Bobo-Dioulasso	Bobo-Dioulasso	Burkina Faso	Submitted
D7BPLGML	*C. imicola*	Gao	Gao	Mali	Submitted
A2HSWZW	*C. imicola*	Swimuwini	Harare	Zimbabwe	Submitted
B2DMAMG	*C. imicola*	Masoandro	Toamasina	Madagascar	Submitted
B5CMAMZ	*C. imicola*	Maputo	Maputo	Mozambique	Submitted
G6GBEN	*C. imicola*	Ouidah	Atlantique	Benin	Submitted
ZA22	*C. isioloensis*	Port Elizabeth	Eastern Cape	South Africa	Submitted
ZA23	*C. isioloensis*	Port Elizabeth	Eastern Cape	South Africa	Submitted
ZAKR07	*C. kanagai*	Kruger	Mpumalanga	South Africa	Submitted
REBG06	*C. kibatiensis*	Le Tampon	Saint-Denis	France-Reunion Island	Submitted
D4ASALSEN	*C. kingi*	Salikegne	Kolda	Senegal	Submitted
G7FBOGCAM	*C. kingi*	Bokke Garoua	Garoua	Cameroon	Submitted
C7BSARSEN	*C. kingi*	Saraya	Kedougou	Senegal	Submitted
D3BSARSEN	*C. kingi*	Saraya	Kedougou	Senegal	Submitted
F4DAWPET	*C. kingi*	Awash Park	Afar	Ethiopia	Submitted

(Table S1. Continued)

ID Specimen	Species	Locality	Province	Country	BOLD ID
F4GAWPET	*C. kingi*	Awash Park	Afar	Ethiopia	Submitted
F4EAWPET	*C. kingi*	Awash Park	Afar	Ethiopia	Submitted
ZA41	*C. kwagga*	Pretoria	Gauteng	South Africa	Submitted
ZA42	*C. kwagga*	Pretoria	Gauteng	South Africa	Submitted
GBIS3FAWPET	*C. leucostictus*	Awash Park	Afar	Ethiopia	Submitted
GBIS6FSWMZ	*C. leucostictus*	Maputo	Maputo	Mozambique	Submitted
GBIS6ESWMZ	*C. leucostictus*	Maputo	Maputo	Mozambique	Submitted
ZAKR01	*C. loxodontis*	Kruger	Mpumalanga	South Africa	Submitted
ZAKR02	*C. loxodontis*	Kruger	Mpumalanga	South Africa	Submitted
ZA25	*C. macintoshi*	Port Elizabeth	Eastern Cape	South Africa	Submitted
ZA26	*C. macintoshi*	Port Elizabeth	Eastern Cape	South Africa	Submitted
ZA24	*C. macintoshi*	Port Elizabeth	Eastern Cape	South Africa	Submitted
GBIS7DMKKEN	*C. magnus*	Munukathi	Kitui	Kenya	Submitted
GBIS7FMKKEN	*C. magnus*	Munukathi	Kitui	Kenya	Submitted
GBIS7GMKKEN	*C. magnus*	Munukathi	Kitui	Kenya	Submitted
GBIS4FAWPET	*C. magnus*	Awash Park	Afar	Ethiopia	Submitted
GBIS4GAWPET	*C. magnus*	Awash Park	Afar	Ethiopia	Submitted
GBIS7EMKKEN	*C. magnus*	Munukathi	Kitui	Kenya	Submitted
D8FPLGML	*C. milnei*	Gao	Gao	Mali	Submitted
D11ABOGCAM	*C. miombo*	Bokke Garoua	Garoua	Cameroon	Submitted
D3HSALSEN	*C. miombo*	Salikegne	Kolda	Senegal	Submitted
E9HMKKEN	*C. miombo*	Munukathi	Kitui	Kenya	Submitted
D4BSALSEN	*C. miombo*	Salikegne	Kolda	Senegal	Submitted
D4CSALSEN	*C. miombo*	Salikegne	Kolda	Senegal	Submitted
E5CMKKEN	*C. miombo*	Munukathi	Kitui	Kenya	Submitted
G5GBEN	*C. miombo*	Ouidah	Atlantique	Benin	Submitted
G5HBEN	*C. miombo*	Ouidah	Atlantique	Benin	Submitted
BFBB01	*C. miombo*	Bobo-Dioulasso	Bobo-Dioulasso	Burkina Faso	Submitted
G5BBEN	*C. miombo*	Ouidah	Atlantique	Benin	Submitted
G5DBEN	*C. miombo*	Ouidah	Atlantique	Benin	Submitted
G6EBEN	*C. miombo*	Ouidah	Atlantique	Benin	Submitted
KEN56	*C. miombo*	Munukathi	Kitui	Kenya	Submitted
C1FMBSEN	*C. moreli*	Mbao	Dakar	Senegal	Submitted
C1CMBSEN	*C. moreli*	Mbao	Dakar	Senegal	Submitted
C1AMBSEN	*C. moreli*	Mbao	Dakar	Senegal	Submitted
C1BMBSEN	*C. moreli*	Mbao	Dakar	Senegal	Submitted
C1DMBSEN	*C. moreli*	Mbao	Dakar	Senegal	Submitted
C1EMBSEN	*C. moreli*	Mbao	Dakar	Senegal	Submitted
B7ANGSEN	*C. murphyi*	Niague	Dakar	Senegal	Submitted
B7BNGSEN	*C. murphyi*	Niague	Dakar	Senegal	Submitted

(Table S1. Continued)

ID Specimen	Species	Locality	Province	Country	BOLD ID
B7DNGSEN	*C. murphyi*	Niague	Dakar	Senegal	Submitted
B7FNGSEN	*C. murphyi*	Niague	Dakar	Senegal	Submitted
B7GNGSEN	*C. murphyi*	Niague	Dakar	Senegal	Submitted
B7HNGSEN	*C. murphyi*	Niague	Dakar	Senegal	Submitted
B8APHSEN	*C. murphyi*	Parc de Hann	Dakar	Senegal	Submitted
B8BPHSEN	*C. murphyi*	Parc de Hann	Dakar	Senegal	Submitted
B8DPHSEN	*C. murphyi*	Parc de Hann	Thies	Senegal	Submitted
B8EPHSEN	*C. murphyi*	Parc de Hann	Thies	Senegal	Submitted
B8FPHSEN	*C. murphyi*	Parc de Hann	Thies	Senegal	Submitted
B8GPHSEN	*C. murphyi*	Parc de Hann	Thies	Senegal	Submitted
B8HPHSEN	*C. murphyi*	Parc de Hann	Thies	Senegal	Submitted
B9GMBSEN	*C. murphyi*	Mbao	Dakar	Senegal	Submitted
B9HMBSEN	*C. murphyi*	Mbao	Dakar	Senegal	Submitted
GBIS6GBOGCAM	*C. neavei*	Bokke Garoua	Garoua	Cameroon	Submitted
ZA52	*C. neavei*	Pretoria	Gauteng	South Africa	Submitted
C6HSARSEN	*C. nevilli*	Saraya	Kedougou	Senegal	Submitted
B1AMAMG	*C. nevilli*	Masoandro	Toamasina	Madagascar	Submitted
G2FBOGCAM	*C. nevilli*	Bokke Garoua	Garoua	Cameroon	Submitted
G7DBOGCAM	*C. nevilli*	Bokke Garoua	Garoua	Cameroon	Submitted
C6GSARSEN	*C. nevilli*	Saraya	Kedougou	Senegal	Submitted
B1BMAMG	*C. nevilli*	Masoandro	Toamasina	Madagascar	Submitted
B1CMAMG	*C. nevilli*	Masoandro	Toamasina	Madagascar	Submitted
B1DMAMG	*C. nevilli*	Masoandro	Toamasina	Madagascar	Submitted
B3FMAMG	*C. nevilli*	Masoandro	Toamasina	Madagascar	Submitted
GBIS1APLGML	*C. nivosus*	Gao	Gao	Mali	Submitted
GBIS1BMBSEN	*C. nivosus*	Mbao	Dakar	Senegal	Submitted
GBIS1CMBSEN	*C. nivosus*	Mbao	Dakar	Senegal	Submitted
GBIS1DMBSEN	*C. nivosus*	Mbao	Dakar	Senegal	Submitted
GBIS5HSWMZ	*C. nivosus*	Maputo	Maputo	Mozambique	Submitted
ZA53	*C. ovalis*	Nelspruit	Mpumalanga	South Africa	Submitted
C10DSARSEN	*C. oxystoma*	Saraya	Kedougou	Senegal	Submitted
C12DSARSEN	*C. oxystoma*	Saraya	Kedougou	Senegal	Submitted
C10FSARSEN	*C. oxystoma*	Saraya	Kedougou	Senegal	Submitted
C11ASARSEN	*C. oxystoma*	Saraya	Kedougou	Senegal	Submitted
C4HMBSEN	*C. oxystoma*	Mbao	Dakar	Senegal	Submitted
C5DMBSEN	*C. oxystoma*	Mbao	Dakar	Senegal	Submitted
C9BSARSEN	*C. oxystoma*	Saraya	Kedougou	Senegal	Submitted
D8DPLGML	*C. oxystoma*	Gao	Gao	Mali	Submitted
C12HMBSEN	*C. oxystoma*	Mbao	Dakar	Senegal	Submitted
C5BMBSEN	*C. oxystoma*	Mbao	Dakar	Senegal	Submitted

(Table S1. Continued)

ID Specimen	Species	Locality	Province	Country	BOLD ID
D7GPLGML	*C. oxystoma*	Gao	Gao	Mali	Submitted
C4GMBSEN	*C. oxystoma*	Mbao	Dakar	Senegal	Submitted
C5AMBSEN	*C. oxystoma*	Mbao	Dakar	Senegal	Submitted
C5FMBSEN	*C. oxystoma*	Mbao	Dakar	Senegal	Submitted
G4APLGML	*C. oxystoma*	Gao	Gao	Mali	Submitted
G9FBEN	*C. oxystoma*	Ouidah	Atlantique	Benin	Submitted
B8CPOSEN	*C. pseudopallidipennis*	Pout	Thies	Senegal	Submitted
C2ATHSEN	*C. pseudopallidipennis*	Thies	Thies	Senegal	Submitted
C2BTHSEN	*C. pseudopallidipennis*	Thies	Thies	Senegal	Submitted
C2DTHSEN	*C. pseudopallidipennis*	Thies	Thies	Senegal	Submitted
C2FTHSEN	*C. pseudopallidipennis*	Thies	Thies	Senegal	Submitted
C3BTHSEN	*C. pseudopallidipennis*	Thies	Thies	Senegal	Submitted
C3CTHSEN	*C. pseudopallidipennis*	Thies	Thies	Senegal	Submitted
C3GTHSEN	*C. pseudopallidipennis*	Thies	Thies	Senegal	Submitted
C5HTHSEN	*C. pseudopallidipennis*	Thies	Thies	Senegal	Submitted
G5EBEN	*C. pseudopallidipennis*	Ouidah	Atlantique	Benin	Submitted
BJTR13	*C. pseudopallidipennis*	Tori-Bossito	Atlantique	Benin	Submitted
BJTR14	*C. pseudopallidipennis*	Tori-Bossito	Atlantique	Benin	Submitted
G5FBEN	*C. pseudopallidipennis*	Ouidah	Atlantique	Benin	Submitted
GBIS5EMKKEN	*C. pycnostictus*	Munukathi	Kitui	Kenya	Submitted
GBIS6CMKKEN	*C. pycnostictus*	Munukathi	Kitui	Kenya	Submitted
A8DSWZW	*C. ravus*	Swimuwini	Harare	Zimbabwe	Submitted
F3FAWPET	*C. ravus*	Awash Park	Afar	Ethiopia	Submitted
A8FSWZW	*C. ravus*	Swimuwini	Harare	Zimbabwe	Submitted
A8GSWZW	*C. ravus*	Swimuwini	Harare	Zimbabwe	Submitted
B3HMAMZ	*C. schultzei*	Maputo	Maputo	Mozambique	Submitted
B4FMAMZ	*C. schultzei*	Maputo	Maputo	Mozambique	Submitted
C12CSARSEN	*C. similis*	Saraya	Kedougou	Senegal	Submitted
A10APHSEN	*C. similis*	Parc de Hann	Dakar	Senegal	Submitted
C4CMBSEN	*C. similis*	Mbao	Dakar	Senegal	Submitted
C4DMBSEN	*C. similis*	Mbao	Dakar	Senegal	Submitted
C4EMBSEN	*C. similis*	Mbao	Dakar	Senegal	Submitted
ZA27	*C. similis*	Port Elizabeth	Eastern Cape	South Africa	Submitted
ZA29	*C. similis*	Port Elizabeth	Eastern Cape	South Africa	Submitted
D2DSARSEN	*C. sp. #20*	Saraya	Kedougou	Senegal	Submitted
D2ESARSEN	*C. sp. #20*	Saraya	Kedougou	Senegal	Submitted
D2FSARSEN	*C. sp. #20*	Saraya	Kedougou	Senegal	Submitted
D2GSARSEN	*C. sp. #20*	Saraya	Kedougou	Senegal	Submitted
D3ASARSEN	*C. sp. #20*	Saraya	Kedougou	Senegal	Submitted
D2HSARSEN	*C. sp. #20*	Saraya	Kedougou	Senegal	Submitted

(Table S1. Continued)

ID Specimen	Species	Locality	Province	Country	BOLD ID
E4BMKKEN	*C. sp. #22*	Munukathi	Kitui	Kenya	Submitted
E4EMKKEN	*C. sp. #22*	Munukathi	Kitui	Kenya	Submitted
E4FMKKEN	*C. sp. #22*	Munukathi	Kitui	Kenya	Submitted
E4HMKKEN	*C. sp. #22*	Munukathi	Kitui	Kenya	Submitted
E4CMKKEN	*C. sp. #22*	Munukathi	Kitui	Kenya	Submitted
ZA15	*C. sp. #54df*	Port Elizabeth	Eastern Cape	South Africa	Submitted
B5AMAMZ	*C. subschultzei*	Maputo	Maputo	Mozambique	Submitted
B6CMAMZ	*C. subschultzei*	Maputo	Maputo	Mozambique	Submitted
A9AMAZW	*C. subschultzei*	Malipati	Harare	Zimbabwe	Submitted
B4DMAMZ	*C. subschultzei*	Maputo	Maputo	Mozambique	Submitted
B5HMAMZ	*C. subschultzei*	Maputo	Maputo	Mozambique	Submitted
B6AMAMZ	*C. subschultzei*	Maputo	Maputo	Mozambique	Submitted
GBIS10H	*C. tororoensis*	Kruger	Mpumalanga	South Africa	Submitted
GBIS11D	*C. tororoensis*	Kruger	Mpumalanga	South Africa	Submitted
ZA19	*C. tropicalis*	Port Elizabeth	Eastern Cape	South Africa	Submitted
ZA20	*C. tropicalis*	Port Elizabeth	Eastern Cape	South Africa	Submitted
ZA21	*C. tropicalis*	Port Elizabeth	Eastern Cape	South Africa	Submitted
A7GMAZW	*C. tuttifrutti*	Malipati	Harare	Zimbabwe	Submitted
A7EMAZW	*C. tuttifrutti*	Malipati	Harare	Zimbabwe	Submitted
A7HMAZW	*C. tuttifrutti*	Malipati	Harare	Zimbabwe	Submitted
ZAPR06	*C. tuttifrutti*	Pretoria	Gauteng	South Africa	Submitted
B2BMAMG	*C. zuluensis*	Masoandro	Toamasina	Madagascar	Submitted
B2CMAMG	*C. zuluensis*	Masoandro	Toamasina	Madagascar	Submitted
B3DMAMG	*C. zuluensis*	Masoandro	Toamasina	Madagascar	Submitted
F1DMKKEN	*C. zuluensis*	Munukathi	Kitui	Kenya	Submitted
B2AMAMG	*C. zuluensis*	Masoandro	Toamasina	Madagascar	Submitted
B3CMAMG	*C. zuluensis*	Masoandro	Toamasina	Madagascar	Submitted
ZA6	*C. zuluensis*	Port Elizabeth	Eastern Cape	South Africa	Submitted
ZA7	*C. zuluensis*	Port Elizabeth	Eastern Cape	South Africa	Submitted

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Discussion générale

Les objectifs de cette thèse étaient (i) de réviser le schéma systématique et taxonomique du genre *Culicoides* en région Afrotropicale en ciblant les sous-genres et les groupes d'intérêt vétérinaire, et (ii) d'améliorer les connaissances sur la bio-écologie des espèces au Sénégal en caractérisant le comportement trophique des espèces les plus abondantes et en décrivant à l'espèce les habitats larvaires et la dynamique des populations immatures. Pour répondre à ces objectifs, nous avons réalisé des phylogénies moléculaires multi-marqueurs avec une représentation de la diversité sans précédent dans la littérature (33 espèces) (chapitre 2, article 1) et fait des délimitations d'espèces avec une approche de taxonomie intégrative (données morphologique et données moléculaires). Une fois le cadre systématique et taxonomique révisé, nous avons utilisé ces nouvelles connaissances taxonomiques pour compléter l'état des connaissances bio-écologiques des populations adultes et immatures. L'approche originale en anneaux pour l'étude du comportement trophique des espèces abondantes dans la région des Niayes, Sénégal, a permis de caractériser à l'espèce, le comportement trophique d'espèces vectrices en fonction de la disponibilité d'hôtes (chapitre 3, article 2). Enfin, la constitution d'une base de données de séquences barcodes de référence (chapitre 4, article 5) couvrant une large partie de la diversité culicoidienne afrotropicale a permis de décrire de façon plus précises les habitats larvaires des espèces abondantes dans des environnements équins et de caractériser la dynamique de leurs populations (chapitre 4, article 3 et 4). Les principaux résultats de cette thèse complètent, mettent à jour et approfondissent le corpus de connaissances sur la diversité des *Culicoides* présents en région Afrotropicale avec un focus sur l'Afrique de l'Ouest, travail indispensable pour l'élaboration de stratégies de contrôle et de gestion des populations.

1. Un schéma systématique et taxonomique partiellement validé

Avant nos travaux, les études de taxonomie réalisées en Afrique australe, occidentale et orientale étaient basées exclusivement sur la morphologie (Bakhoum *et al.*, 2013; Cornet et Brunhes, 1994; Glick, 1990; Labuschagne, 2016; Meiswinkel, 1996). Nos résultats appuyés sur 4 marqueurs moléculaires nous permettent pour la première fois de proposer un nouveau schéma systématique validé (Table 8) où :

- le groupe Imicola, sous-genre *Avaritia*, est monophylétique. Il inclut *C. bolitinos*, *C. imicola*, *C. kwagga*, *C. loxodontis*, *C. miombo*, *C. pseudopallidipennis*, *C.*

tuttifrutti et une nouvelle espèce non-décrite nommée *C. sp*. #22. Ceci confirme les études réalisées précédemment (Bellis *et al.*, 2014; Labuschagne, 2016; Mathieu, 2011) .

- le groupe Dasyops, sous-genre *Avaritia*, composé de *C. alticola*, *C. dasyops* et *C. kanagai*, compte une nouvelle espèce non-décrite nommée *C. sp.* #54.
- le groupe Milnei (sans affiliation subgénérique) décrit par Cornet et al. (1974) est monophylétique et pourrait être proche du sous-genre *Avaritia*. Une affiliation subgénérique de ce groupe ou la création d'un sous-genre propre nécessite des études complémentaires.
- le groupe Similis appartient au sous-genre *Synhelea* comme répertorié par (Borkent, 2016) avec une affiliation dans le même sous-genre du groupe Neavei (représenté dans notre jeu de données par *C. neavei* et *C. ovalis*) (sans affiliation subgénérique pour Borkent, 2016).
- Le sous-genre *Remmia* et son unique groupe d'espèces, le groupe Schultzei est monophylétique. Les délimitations moléculaires montrent une divergence entre les populations de *C. oxystoma* du Liban et celles du Mali et du Sénégal. Des études antérieures avaient déjà suggéré la possible existence de diversité cryptique au sein de *C. oxystoma* (Bakhoum *et al.*, 2013; Cornet et Brunhes, 1994; Harrup *et al.*, 2016).

Tableau 8: La classification subgénérique utilisée par Borkent (2016), Meiswinkel (1996), Labuschagne (2016) et dans cette thèse, Bakhoum (2017)

	Borkent (2016)		Meiswinkel (1996)		Labuschagne (2016)		Bakhoum (2017)	
Espèce	**Sous-genre**	**Groupe**	**Sous-genre**	**Groupe**	**Sous-genre**	**Groupe**	**Sous-genre**	**Groupe**
C. alticola	*Avaritia*	-	*Avaritia*	Dasyops	*Avaritia*	Dasyops	*Avaritia*	Dasyops
C. austeni	-	Milnei	*Hoffmania*	Milnei	-	Milnei	-	Milnei
C. bolitinos	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola
C. candolfii	*Avaritia*	-	*Avaritia*	-	*Avaritia*	-	*Avaritia*	-
C. dasyops	*Avaritia*	-	*Avaritia*	Dasyops	*Avaritia*	Dasyops	*Avaritia*	Dasyops
C. enderleini	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei
C. exspectator	*Synhelea*	Similis	-	Similis	-	Similis	*Synhelea*	Similis
C. grahamii	*Avaritia*	-	*Avaritia*	Grahamii	*Avaritia*	Grahamii	*Avaritia*	Grahamii
C. gulbenkiani	*Avaritia*	-	*Avaritia*	Gulbenkiani	*Avaritia*	Gulbenkiani	*Avaritia*	Gulbenkiani
C. imicola	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola
C. isioloensis	-	Milnei	*Hoffmania*	Milnei	-	Milnei	-	Milnei
C. kanagai	*Avaritia*	-	*Avaritia*	Dasyops	*Avaritia*	Dasyops	*Avaritia*	Dasyops
C. kibatiensis	*Avaritia*	-	*Avaritia*	-	*Avaritia*	-	*Avaritia*	-
C. kingi	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei
C. kwagga	-	-	-	-	*Avaritia*	Imicola	*Avaritia*	Imicola
C. loxodontis	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola
C. miombo	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola
C. milnei	-	Milnei	-	Milnei	-	Milnei	-	Milnei
C. moreli	-	Milnei	*Hoffmania*	Milnei	-	Milnei	-	Milnei
C. neavi	-	Neavei	-	Neavei	-	Neavei	*Synhelea*	Neavei
C. nevilli	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei
C. ovalis	-	Neavei	-	Neavei	-	Neavei	*Synhelea*	Neavei
C. oxystoma	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei
C. pseudopallidipennis	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola
C. ravus	*Synhelea*	Similis	-	Similis	-	Similis	*Synhelea*	Similis
C. schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei
C. similis	*Synhelea*	Similis	-	Similis	-	Similis	*Synhelea*	Similis
C. sp. # 20	-	-	-	-	-	-	*Avaritia*	Orientalis
***C. sp.* # 22**	-	-	-	-	-	-	*Avaritia*	Imicola
***C. sp.* # 54**	-	-	-	-	*Avaritia*	Dasyops	*Avaritia*	Dasyops
C. subchultzei	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei	*Remmia*	Schultzei
C. trifasciellus	*Avaritia*	-	*Avaritia*	Orientalis	*Avaritia*	Orientalis	*Avaritia*	Orientalis
C. tropicalis	*Synhelea*	-	*Synhelea*	Tropicalis	*Synhelea*	Tropicalis	*Synhelea*	Tropicalis
C. tuttifrutti	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola	*Avaritia*	Imicola
C. zuluensis	-	Milnei	*Hoffmania*	Milnei	-	Milnei	-	Milnei

En gras = nouvelle espèce pour la science

2. Des comportements trophiques mieux caractérisés vis-à-vis des chevaux

La caractérisation des préférences trophiques des espèces vectrices ou suspectées vectrices est un facteur clé pour comprendre la transmission des pathogènes. Le risque de transmission est dépendant de plusieurs facteurs dont le taux de piqûre, les préférences trophiques et le taux de parturité (Macdonald, 1950). Chez les arthropodes hématophages, le choix de l'hôte est déterminé par des facteurs génétiques que l'on connaît peu, des facteurs écologiques et la disponibilité de l'hôte (Gillies, 1972; Takken et Verhulst, 2013). Les différentes méthodes utilisées pour évaluer la préférence trophique des *Culicoides*, que ce soit des approches directes (Fall *et al.*, 2015a; Fall *et al.*, 2015b; Viennet *et al.*, 2011; Viennet *et al.*, 2012) ou indirectes (Garros *et al.*, 2011; Lassen *et al.*, 2012) ne prenent pas en compte la distribution des hôtes potentiels présents sur le site, en dehors des hôtes appâts.

La zone des Niayes au Sénégal est une bande étroite de 180 kilomètres de long sur 25-30 kilomètres de large, à l'Ouest du Sénégal, à proximité de Dakar. C'est une zone d'élevage importante hébergeant environ 90,000 bovins, 179,000 ovins et caprins et 17,100 chevaux (Bouyer *et al.*, 2014; Fall et Badiane, 2001; Fall *et al.*, 2000). L'élevage dans cette zone a une dominante intensive ou semi-intensive (Fall *et al.*, 2000). La présence et l'abondance de *C. imicola*, *C. kingi* et *C. oxystoma* dans la zone des Niayes (Diarra *et al.*, 2014; Fall *et al.*, 2015a; Fall *et al.*, 2015c) démontrées récemment pose la question du rôle de ces espèces dans la transmission du virus de la peste équine (Akakpo *et al.*, 2011; Diouf *et al.*, 2012), mais aussi des virus de la FCO ou de l'EHD.

Nos travaux dans la zone des Niayes montrent que *C. oxystoma* et *C. imicola* préfèrent principalement les chevaux, contrairement à *C. kingi* qui favorise les bovins mais se gorge également sur des chevaux en l'absence de bovins. Son comportement serait donc plus opportuniste. Des études antérieures de préférences trophiques sur appât cheval et mouton ont montré que *C. oxystoma* et *C. imicola* avaient une préférence pour les chevaux par rapport aux moutons (Fall *et al.*, 2015c). De plus, El Sinnary et al. (1985) ont reporté que *C. kingi* était attiré par les bovins au nord du Soudan. La dispersion active, pour la recherche du repas de sang, de *C. imicola*, *C. kingi* et *C. oxystoma* montre des patrons différents avec des conséquences épidémiologiques. La dispersion active de *C. imicola* et *C. oxystoma* est d'environ 200 mètres, contrairement à celle de *C. kingi* qui peut atteindre 2,000 mètres. Ainsi, en plus d'être opportuniste, *C. kingi* a une distance de vol active plus importante. En région Paléarctique, il a été reporté récemment pour des especes européennes d'intérêt vétérinaire des

distances de vol sur terre similaires avec différentes méthodes (Kluiters *et al.*, 2015 ; Kirkeby *et al.*, 2013). Il est par ailleurs connus que les *Culicoides* sont capables de voler plusieurs centaines de kilomètres avec un vol passif aidé par le vent (Burgin *et al.*, 2013; Jacquet *et al.*, 2015).

Ces éléments nouveaux pour *C. kingi* ajoutés à son abondance importante et sa large répartition au Sénégal (Diarra *et al.*, 2014; Fall *et al.*, 2015a; Fall *et al.*, 2015c) et à son comportement trophique (Fall *et al.*, 2015c) justifient qu'on concentre les futurs efforts de recherche afin de déterminer le rôle épidémiologique de cette espèce dans la transmission des souches circulantes de peste équine en Afrique de l'Ouest. Il serait aussi important de renouveler cette étude avec une approche en anneaux dans d'autres environnements avec des importances relatives d'hôtes différents (moins de chevaux et plus de ruminants) à différentes périodes de l'année avec les mêmes espèces de *Culicoides* afin de vérifier nos hypothèses et de décrire la dynamique saisonnière du comportement trophique et de la dispersion active.

3. Gestion et lutte antivectorielle : quelles options pour les populations immatures ?

Les épisodes de peste équine de 2007 au Sénégal ont montré, s'il le fallait, les enjeux sanitaires et économiques de la transmission de virus transmis par les *Culicoides* dans la région des Niayes. Ces épisodes questionnent sur les stratégies de gestion et de contrôle des populations de *Culicoides* en période d'épizootie et inter-épidémique.

Les données actuelles montrent que les *Culicoides* sont très sensibles aux molécules insecticides utilisées en lutte antivectorielle (Venail, 2015; Venail *et al.*, 2015). Les populations adultes peuvent être ciblées à travers l'utilisation de formulation pour-on appliquées sur le bétail mais dont la rémanence reste généralement faible (Venail, 2015; Venail *et al.*, 2011). De plus, les essais de terrain sur la sensibilité et la rémanence des produits commerciaux sont réalisés sur ovins (Venail, 2015) et aucun essai à ce jour n'a été réalisé sur cheval, *a fortiori* en milieu tropical. Par ailleurs, le cheval est réputé pour être un animal avec une transpiration importante qui lessive les formulations déposées sur sa ligne dorsale et avec une sensibilité dermatologique forte, limitant les applications topiques de produits chimiques. Ainsi, la gestion environnementale des écuries et des élevages pour réduire le nombre d'habitats larvaires et contrôler les populations immatures apparaît comme une option raisonnable.

Différentes méthodes sont utilisées pour identifier les habitats larvaires de *Culicoides*: (i) mettre des pièges à émergence sur les habitats potentiels *in situ* et collecter les adultes de

Culicoides émergés de ces habitats (Dipeolu et Ogunrinade, 1976; Harrup *et al.*, 2013; Jenkins et Young, 2010), (ii) collecter des échantillons de substrat à partir des habitats larvaires et les conserver au laboratoire pendant plusieurs semaines jusqu'à l'émergence des *Culicoides* adultes (Uslu et Dik, 2007; Zimmer *et al.*, 2008), et (iii) utiliser la technique de flottaison pour pouvoir prélever les larves ou les nymphes (Blackwell et King, 1997; Kettle, 1975; Uslu et Dik, 2006). Les deux premières méthodes ne sont pas adaptées à l'identification rapide, en raison de l'émergence plus ou moins tardive des *Culicoides* et des efforts de maintenance nécessaire pour incuber les échantillons jusqu'à l'émergence. Pour la dernière méthode, la difficulté réside dans l'absence de clé d'identification morphologique pour les larves de *Culicoides*. L'utilisation d'une approche de taxonomie intégrative avec une base de données moléculaire de référence pour 42 espèces afro-tropicales de *Culicoides* a permis de contourner ce problème.

Il est possible d'envisager une lutte antivectorielle ciblée sur les stades immatures sous certaines conditions (Carpenter *et al.*, 2008; Mullens *et al.*, 2008). Il est nécessaire dans un premier temps de bien connaitre les habitats larvaires. La revue de la littérature décrit différents habitats larvaires pour les *Culicoides* notamment en zone paléarctique (Blackwell *et al.*, 1994; Dipeolu et Ogunrinade, 1976; Jenkins et Young, 2010; Nevill, 1967; Yanase *et al.*, 2013; Zimmer *et al.*, 2014). Ils se répartissent en quatre types : (i) les substrats aquatiques et semi-aquatiques d'eau douce, d'eau salée ou saumâtre qui alimentent les étangs, mares, rives, rivières, canaux d'irrigation, flaques, ou marais, (ii) les substrats terrestres correspondant aux prairies et cultures, trous d'arbres, (iii) les souches et cactus en décomposition, et (iv) les substrats liés à l'élevage correspondant au tas de fumier, à l'ensilage, à la paille ou aux bouses des grands animaux herbivores.

La première étape de notre travail s'est concentré sur la localisation des habitats larvaires des espèces de *Culicoides* d'intérêt vétérinaire dans des écuries ou des centres équestres de la zone des Niayes. Notre suivi autour et à proximité des centres équestres montre que les habitats des populations d'immatures correspondent à des substrats aquatiques d'eau douce et d'eau salée/saumâtre : bords des lacs, mares et flaques d'eau. De façon inattendue et contre notre hypothèse de travail, aucune larve de *Culicoides* n'a été retrouvé dans les excréments des chevaux, dans la litière à l'intérieur des boxes ni dans les bouses de ruminants échantillonnés. Ceci est dû probablement aux perturbations mécaniques des gîtes potentiels d'oviposition lorsque les boxes et les étables sont nettoyés quotidiennement. En

effet, la litière et les bouses sont utilisées par les agriculteurs pour la fertilisation du sol (agents des centres équestres, communication personnelle).

Pour compléter cette caractérisation des habitats larvaires, un suivi annuel de quatorze habitats larvaires favorables aux *Culicoides* a permis de mesurer six paramètres physicochimiques (taux de carbone, matière organique, matière sèche, salinité, pH et conductivité de l'eau). Les analyses montrent que ces paramètres physico-chimiques sont restés modérément stables pendant la période de l'étude et que le pH, la salinité et le taux de matière organique sont les paramètres qui permettent de mieux décrire la présence de larves. Certaines espèces sont capable d'exploiter une large gamme d'habitats larvaires : c'est le cas de *C. nivosus* et *C. oxystoma* que l'on a retrouvé dans tous les types habitats identifiés que ce soit les bords de mares, pourtours de flaques et bords de lacs d'eau douce ainsi que lacs d'eau saumâtre. Ces milieux sont caractérisés par des paramètres allant du pH accide à basique, de forte variation de la teneur en sel, tout comme pour la matière organique. Cette aptitude à se développer dans une multitude d'habitats indique que ces espèces sont relativement plastiques vis à vis des conditions du milieu et peuvent donc potentiellement être retrouvée dans tous les milieux humides. D'autres par contre sont écologiquement très exigeantes, c'est le cas de *C. distinctipennis* et de *C. similis* qui sont présents uniquement dans les habitats larvaires de type bord de lac d'eau douce avec une teneur en matière organique forte et un pH acide ou *C. kingi* présent dans les habitats de type lac salin. Concernant l'espèce *C. imicola* dont les caractéristiques d'habitats larvaires ont été décrits dans la littérature en Europe et en Afrique du Sud (Foxi et Delrio, 2010; Nevill, 1967), nos observations confirment le fait que ses habitats larvaires sont des substrats semi-humides comme le bord des ruisseaux, des mares et flaques d'eau. En Afrique du Sud, *C. imicola* émerge en abondance à partir des prairies humides (Labuschagne, communication personnelle). En effet, la nymphe de cette espèce manque de capacité à flotter à la surface de l'eau et se noie quand son habitat de reproduction est inondé.

Sans surprise les espèces de *Culicoides* semblent posséder des gammes d'habitats larvaires avec des paramètres physicochimiques répondant à leurs besoins (Foxi et Delrio, 2010; Zimmer *et al.*, 2014; Zimmer *et al.*, 2013). Cependant, les facteurs (signaux chimiques, visuels…) impliqués dans la localisation des sites de ponte sont très peu connus et encore peu étudiés (Gonzalez *et al.*, 2013).

Au total, 12 espèces de *Culicoides* ont émergés des substrats prélevés à partir des habitats larvaires. Cependant, le nombre d'espèces de *Culicoides* collectées dans la zone des Niayes en utilisant le piège de référence pour les adultes (piège *Onderstepoort Veterinary Institute*) est supérieur à ce nombre (Diarra *et al.*, 2014; Fall *et al.*, 2015a). Ceci peut s'expliquer par la non-exhaustivité des habitats larvaires identifiés. Egalement, il est connu que le maintien des substrats prélevés au laboratoire peut induire une surmortalité différentielle selon les espèces. En effet, les conditions biotiques et abiotiques des échantillons de substrats varient différemment de l'habitat d'origine en raison de leur taille (absence d'effet tampon de l'habitat) mais également des conditions environnementales et climatiques de l'insectarium qui ne varient pas. Aussi, il est possible que les espèces les plus tolérantes aient été sélectionnées au profit d'espèces plus spécifiques.

4. Quelles recommandations pour les agriculteurs et les centres équestres?

A la lumière de nos résultats, et afin de répondre de façon opérationnelle, nous pouvons émettre les recommandations suivantes :

- Mettre en place des mesures d'assainissement des mares et flaques d'eau aux alentours des centres équestres, voire une éventuelle application ciblée de larvicides sélectifs et peu toxiques pour l'environnement et la faune non-cible ;
- Eliminer les fuites d'eau à l'intérieur et aux alentours des centres équestres;
- Empêcher certaines modifications environnementales ou tout mieux les aménager lorsqu'elles sont indispensables telles que les canaux d'irrigation et les creux d'eau liées à l'intensification des pratiques agricoles et qui pourraient d'autre part favoriser la colonisation et l'abondance de certaines espèces de *Culicoides* ;
- Mettre en place des puits et/ou forages fermés pour que les *Culicoides* n'accèdent pas au bord de l'eau.

Conclusions générales et perspectives

L'ensemble de ce travail confirme à quel point l'approche intégrative est un très bon outil pour réviser la taxonomie et la systématique du genre *Culicoides* en les mettant au service de la biologie et de l'écologie pour des fins opérationnelles. Un nouveau schéma systématique est proposé et pourrait être inclus dans la prochaine révision du genre *Culicoides* par le spécialiste Art Borkent avec lequel nous collaborons. Nos travaux ont permis de caractériser deux nouvelles espèces *C. sp.* # 22 dans le groupe Imicola (sous-genre *Avaritia*)

et *C. sp.* #54 (groupe Dasyops, sous-genre *Avaritia*) qui feront l'objet d'une publication descriptive dédiée.

D'un point de vue bio-écologique, le travail effectué a permis d'évaluer le comportement trophique de trois espèces d'intérêt vétérinaire (*C. imicola*, *C. kingi* et *C. oxystoma*) dans la zone des Niayes au Sénégal et de décrire leurs habitats larvaires. En outre, ce travail peut servir de référence méthodologique pour les entomologistes qui souhaiteraient étudier l'écologie larvaire des espèces de *Culicoides* de leurs régions, ainsi que le comportement trophique des espèces d'intérêt.

Annexe

Liste des valorisations

Articles

-Bakhoum M.T., Sarr M., Fall A. G., Ciss M., Labuschagne K., Seck M.T., Fall M., Dicko A., Balenghien T., Bouyer J., Garros C., Baldet T., Gimonneau G. Physicochemical characteristics and *Culicoides* diversity of larval habitats in the Niayes area of Senegal. ***In preparation***

- Bakhoum M.T., Sarr M., Fall A. G., Huber K., Labuschagne K., Gardès L., Fall M., Seck M.T., Gimonneau G., Bouyer J., Baldet T., Garros C. DNA barcoding for *Culicoides* biting midges (Diptera: Ceratopogonidae) and application for *Culicoides* larvae identification in the Niayes area of Senegal, West Africa**. *In preparation***

- **Bakhoum M.T.**, Labuschagne K., Huber K., Fall M., Mathieu B., Venter G., Gardès L., Baldet T., Bouyer J., Fall A.G., Gimonneau G., Garros C. Phylogenetic relationships and molecular delimitation of *Culicoides* Latreille (Diptera: Ceratopogonidae) species in the Afrotropical region: interest for the *Avaritia* subgenus. Submitted to ***Systematic Entomology***

-Bakhoum, M. T., Fall, A. G., Fall, M., Bassene, C. K., Baldet, T., Seck, M. T.*, et al.* (2016). Insight on the larval habitat of Afrotropical *Culicoides* Latreille (Diptera: Ceratopogonidae) in the Niayes area of Senegal, West Africa. ***Parasit Vectors,*** **9**, 462.

-Bakhoum, M. T., Fall, M., Seck, M. T., Gardès, L., Fall, A. G., Diop, M.*, et al.* (2016). Foraging range of arthropods with veterinary interest: New insights for Afrotropical

Culicoides biting midges (Diptera: Ceratopogonidae) using the ring method. ***Acta Trop,*** **157**, 59-67.

Posters

-**Bakhoum MT**, Fall M, Seck MT, Gardès L, Fall AG, Diop M, Mall I, Balenghien T, Baldet T, Gimonneau G, Garros C, Bouyer J. How can we investigate the foraging range of three main *Culicoides* species in the Niayes area of Senegal, West Africa? 20th E-SOVE conference, 3rd-7th October 2016 Lisbon, Portugal

-**Bakhoum MT**, Fall AG, Fall M, Bassene CK, Baldet T, Seck MT, Bouyer J, Garros C and Gimonneau G. *Culicoides* species in the Niayes area of Senegal, West Africa: what are the larval habitats? 20th E-SOVE conference, 3rd-7th October 2016 Lisbon, Portugal

First Record of *Culicoides oxystoma* Kieffer and Diversity of Species within the Schultzei Group of *Culicoides* Latreille (Diptera: Ceratopogonidae) Biting Midges in Senegal : Publication réalisée avant la thèse de Doctorat dans le cadre du Master International en Entomologie Médicale et Vétérinaire, Université de Montpellier (2012)

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First Record of *Culicoides oxystoma* Kieffer and Diversity of Species within the Schultzei Group of *Culicoides* Latreille (Diptera: Ceratopogonidae) Biting Midges in Senegal

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Abstract

The Schultzei group of *Culicoides* Latreille (Diptera: Ceratopogonidae) is distributed throughout Africa to northern Asia and Australasia and includes several potential vector species of livestock pathogens. The taxonomy of the species belonging to this species group is confounded by the wide geographical distribution and morphological variation exhibited by many species. In this work, morphological and molecular approaches were combined to assess the taxonomic validity of the species and morphological variants of the Schultzei group found in Senegal by comparing their genetic diversity with that of specimens from other geographical regions. The species list for Senegal was updated with four species: *Culicoides kingi*, *C. oxystoma*, *C. enderleini* and *C. nevilli* being recorded. This is the first record of *C. oxystoma* from Africa south of Sahara, and its genetic relationship with samples from Israel, Japan and Australia is presented. This work provides a basis for ecological studies of the seasonal and spatial dynamics of species of this species group that will contribute to better understanding of the epidemiology of the viruses they transmit.

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Introduction

The recent introduction and expansion of bluetongue virus (BTV) [1,2] and emergence of Schmallenberg virus [3] in Europe has emphasis the importance of accurate species identification and taxonomy of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) responsible for transmitting these viruses. Currently approximately 1,400 species placed into 29 formal subgenera and 39 informal species groups are recognized worldwide [4,5]. In the Afrotropical region, some 156 species have been described but many more await description [6] and most of the literature on the taxonomy of Afrotropical species are in the need for revision [7–10].

The Afrotropical region is endemic for most of the known *Culicoides*-borne economical important diseases of livestock, like bluetongue virus and epizootic haemorrhagic disease virus (EHDV) [11]. While many local breeds of livestock appear to have achieved some level of tolerance to some of these diseases [12], others, for example African horse sickness virus (AHSV), still cause serious outbreaks in many parts of Africa [13] as recently illustrated with the epidemic recorded in Senegal in 2007 [14,15].

In the Afrotropical region, *Culicoides imicola* Kieffer, subgenus *Avaritia* Fox, is regarded as the most important and proven orbivirus vector species of livestock diseases and this was recently reinforced when this species became an apparent invasive species throughout the Mediterranean basin [16,17]. Other groupings of *Culicoides* which have been implicated in the transmission of some of these viruses is the Schultzei species group. Species belonging to this group have been associated with BTV [18,19], AHSV [20] and EHDV [21,22]. In especially the Australian and Oriental subregions, *C. oxystoma* Kieffer is a well-known vector of bovine

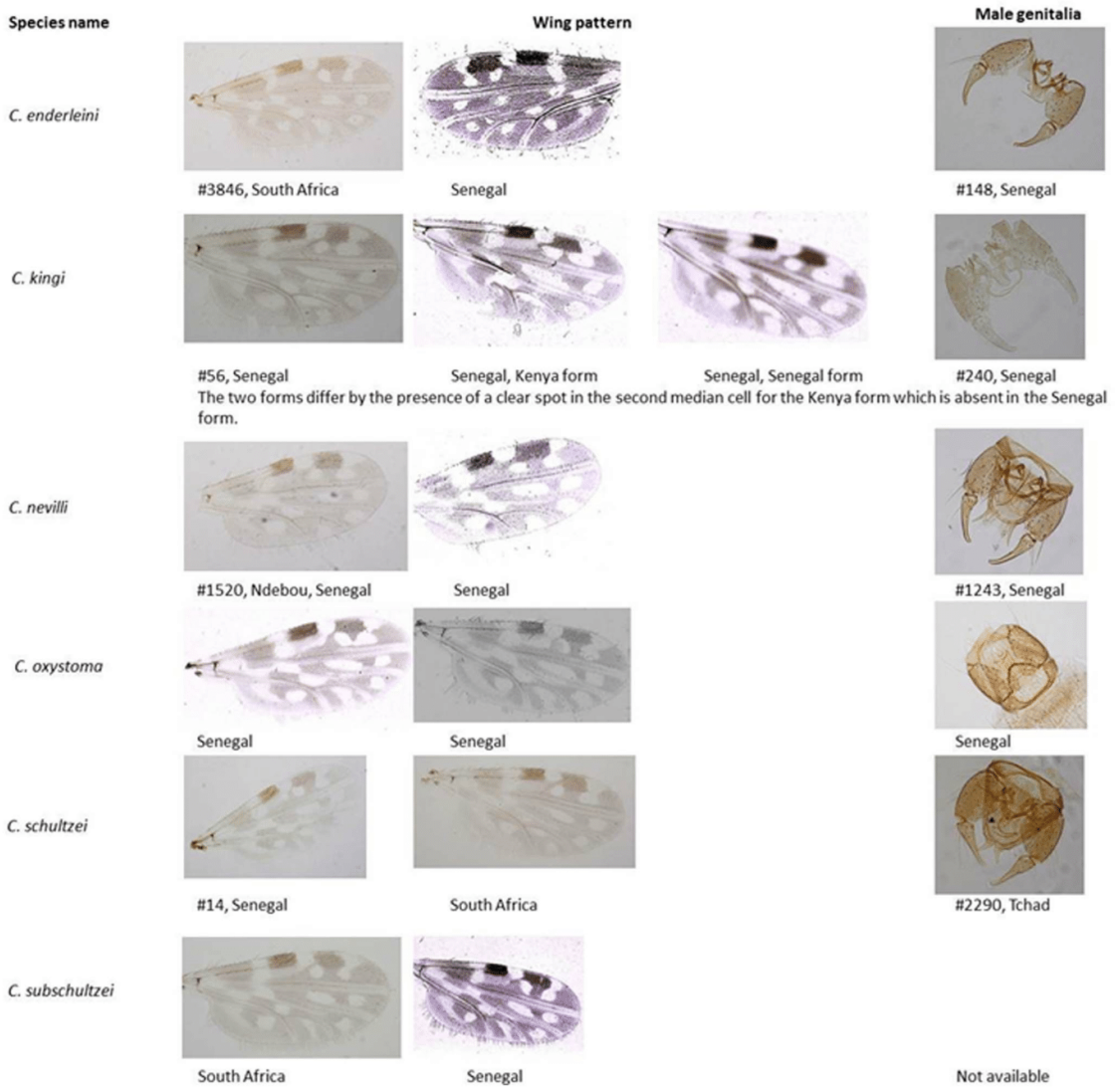

Figure 1. Female wing pattern and male genitalia of the Schultzei species group collected in Senegal (samples from the study and Cornet's collection) and South Africa. The slide reference number refers to the Cornet collection reference numbers [8].
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arboviruses such as Akabane virus [23,24] and is suspected of being vector of EHDV in Israel [25].

The status of the Schultzei group and its subgeneric affiliation has been disputed. Several authors placed this species group within *C.* subg. *Remmia* Glukhova [4,6,26] with regarding this group as a valid subgenus, while others maintain that *C.* subg. *Remmia* is a junior synonym of *C.* subg. *Oecacta* (Poey) [8,27] or leave the group as unplaced to subgenus [28]. The status of the species within this group is equally contentious with widespread use of the name *C. schultzei* for members of the group [29]. The revision of Afrotropical species by Cornet & Brunhes [8] resolved most of the issues associated with the African fauna and Borkent [4] currently places eight species in *C.* subg. *Remmia* (= Schultzei group): *C. schultzei* (Enderlein), *C. oxystoma*, *C. kingi* Austen, *C. rhizophorensis* Khamala and Kettle, *C. neoschultzei* Boorman and Meiswinkel, *C. subschultzei* Cornet and Brunhes, *C. enderleini* Cornet and Brunhes and *C. nevilli* Cornet and Brunhes.

Culicoides oxystoma is known from the Oriental and Australasian regions [28], whereas all the other species within the Schultzei group are confined to the afrotropical region with some species extending north to the Mediterranean and Middle East [8,18,25,28,30–34]. The identity of the most widespread species, currently referred to as *C. oxystoma*, is unclear as many of the records listed by Wirth & Hubert [28] are based on misidentifications of *C. schultzei* and no taxonomic revisions of the Schultzei group have included this species. One barrier to the inclusion of *C.*

Table 1. Female samples used for the molecular analysis, localization and Genbank accession numbers for the COI sequences.

Species	Geographical origin	Genbank accession numbers
C. enderleini	Reunion Island, France, collected in 2005	KF682426–KF682428
C. enderleini	Onderstepoort, South Africa, collected in 2009	KF682478–KF682479
C. enderleini	Madagascar, from Augot et al (2013)	KF186429–KF186431
C. enderleini	Senegal, collected in 2011	KF682471–KF682477
C. kingi	Senegal, collected in 2011	KF682482–KF682495
C. nevilli	Senegal, collected in 2011	KF682496–KF682497
C. nevilli	Madagascar, from Augot et al (2013)	KF186428
C. oxystoma	Senegal, collected in 2011	KF682498–KF682522
C. *oxystoma*	Australia	KF682529–KF682533
C. oxystoma	Israel, from Morag et al (2012)	JN545045–JN545049, JN545052, JN545054
Schultzei Group	Israel, from Morag et al (2012)	JN545050–JN545051, JN545053
C. oxystoma	Japan, from Matsumoto et al (2009)	AB360978, AB360980–AB360985
C. subschultzei	Skukuza Kinger national park, South Africa, collected in 2008	KF682523–KF682525
C. imicola	Reunion Island, France, collected in 2005	KF642480–KF682481

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oxystoma in a revision is that the original description by Kieffer is very brief [35] and the type specimen of *C. oxystoma* from Kolkata (= Calcutta) in India, has been lost [28].

Cornet & Brunhes [8] listed three species belonging to the Schultzei group from Senegal: *C. enderleini*, *C. nevilli* and two morphological forms of *C. kingi* termed a Kenya form and a Senegal form. They separated these latter two forms by the degree of posterior expansion of the basal pale spot basally in cell m_1 of the wing with this spot crossing vein M_2 in the Kenya form but not crossing the vein in the Senegal form (Figure 1). This morphological difference appeared to be associated with a difference in breeding habitat but whether these two forms constitute a single species or not remains unclear. Similarly, the morphological similarity between Oriental *C. oxystoma* and the Afrotropical *C. subschultzei* makes differentiation of these species problematic [36]. *Culicoides schultzei* (= *C. irroratus*) is recorded in southern, eastern and central Africa [8], although it has not been recorded in western Africa [9].

Using molecular analysis of the Cytochrome Oxidase I (COI) gene, Morag et al. [25] recently established that two distinct lineages of the Schultzei group are present in Israel. Not only does this suggest the presence of two species in Israel, one of which is synonymous with the species in Japan, it also suggests that analysis of the COI gene may be useful in addressing some of the taxonomic problems associated with this group, for example the status of the Kenya and Senegal forms of *C. kingi* and that of *C. oxystoma* and *C. subschultzei*. In this work, we combined morphological and molecular analyses to assess the diversity of the species of the Schultzei group present in Senegal in relation to that found in other regions. The specific status of morphological variants of *C. kingi* and *C. oxystoma* present in Senegal was established and an updated species list of Senegal for this group is provided.

Materials and Methods

Specimen Collection

Adult midges were collected using black light/suction traps placed near horses within stud farms at five sites in Senegal (Figure 2) between September and October 2011 (3 nightly trappings/site/month). The owners of stud farms gave permission to conduct the study on their sites. Field workers did not have any contact with the horses. Specimens were preserved in 70% ethanol and identified and sexed under a binocular microscope using the identification keys of Glick [7], Cornet & Brunhes [8] and Boorman [37]. For each species, specimens representing all available morphological variants were included in the analysis. Wings from females and male genitalia were dissected prior to processing and slide-mounted to record these morphological variations. Samples from La Reunion Island (*C. enderleini*), South Africa (*C. enderleini*, *C. subschultzei*, *C. schultzei*), and Australia (*C. oxystoma*) were used to represent the geographic variation of these species. COI sequences submitted by Morag et al [25], Matsumoto et al [38] and Augot et al [39] were added to the dataset (Table 1).

Extraction of Genomic DNA

Genomic DNA was individually extracted following an extraction protocol with Chelex resin in 5% (Resin Chelex100 ®, Chelating Ion Exchange Resin, Bio-Rad, France) as described in Viennet et al [40] and Solano et al [41]. A volume of 500 µl of Chelex solution was dispensed into each tube of 1.5 ml. Each individual was removed from ethanol and dried on blotting paper. The individual was retrieved and transferred to a tube with Chelex solution and ground using a piston. The tubes were incubated at 56°C for 60 minutes and then at 95°C for 30 minutes (for thermal lysis). Immediately after heating, the tubes were centrifuged at 13,000 revs/min for 1 minute to pellet the Chelex resin with inhibitor ions and cellular debris.

Polymerase Chain Reaction Amplification and Sequencing of COI

Cytochrome Oxidase I (COI) amplification of the gene was carried out using primers C1J1718 (Forward) 5′- GGA- GGA-TTT-GGA-AAT-TGA-ATT-GT-3′ and C1N2191 (Reverse) 5′ -CAG-GTA-TTA-AAA-AAA-AAA-TAT-CTT-CTG-G-3′ to obtain an approximately 600 bp product as described previously [42]. Amplification reactions by Polymerase Chain Reaction (PCR) were performed in 25 µl of reaction volume with 5 µl of buffer 5X, 0.5 µl of dNTP (10 mM), 2 µl of MgCl2 (25 mM), 0.5 ml of each primer; 0.5 µl of Taq polymerase (5 U/µl), 15 µl of

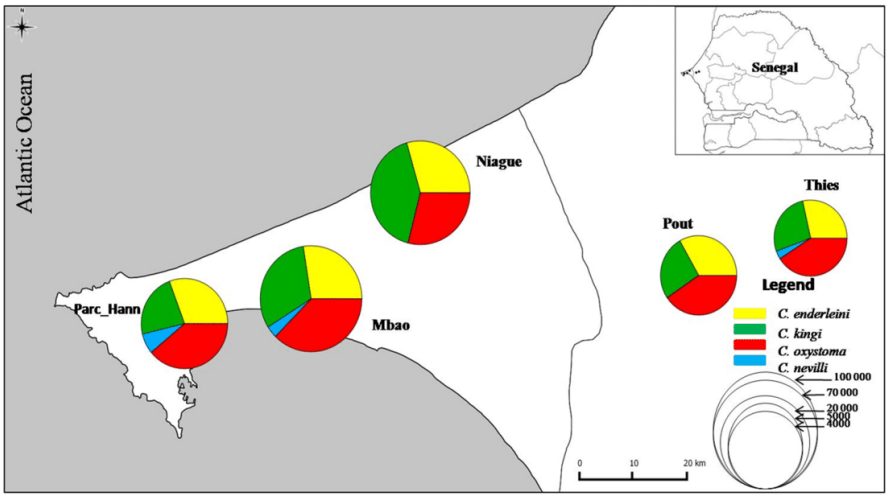

Figure 2. Location of the study sites in Senegal and abundance of species from the Schultzei group.
doi:10.1371/journal.pone.0084316.g002

sterile water and 1 µl of DNA. Touch-up PCR amplification was used to reduce non-specific amplifications and optimize the quality of amplification for better sequencing. The cycling profile of the COI gene consisted of an initial denaturation stage of 1 minute at 94°C, followed by 5 cycles of 40 seconds at 94°C, 40 seconds at 45°C and 1 minute at 72°C, then 35 cycles of 40 seconds at 94°C, 40 seconds at 51°C, 1 minute at 72°C and a final elongation of 5 minutes at 72°C. The PCR products were visualized on 1% agarose gel with a Gel Red staining after migration of 90 minutes at 100 volts by electrophoresis, before being sent to Cogenics (Grenoble, France) for sequencing.

Sequence Alignment, Phylogenetic and Genetic Distance Analyses

Multiple alignments of the sequences were generated using the CLUSTALW algorithm in BioEdit [43]. Molecular evolutionary analyses were conducted using DAMBE [44] and MEGA version 5 [45]. The phylogenetic reconstructions were performed by Maximum Likelihood (ML) and Bayesian analyses (BA). The ML analyses were carried out with MEGA v5 [45], incorporating best fit models of sequence evolution determined using the Akaike Information Criterion (AIC) and employing 1,000 bootstrap replications to determine node reliabilities. The AIC implemented within jModelTest was used to determine the most suitable evolutionary model(s) for the Bayesian and ML analyses [46]. The AIC model selected for the COI was T92+G, followed by

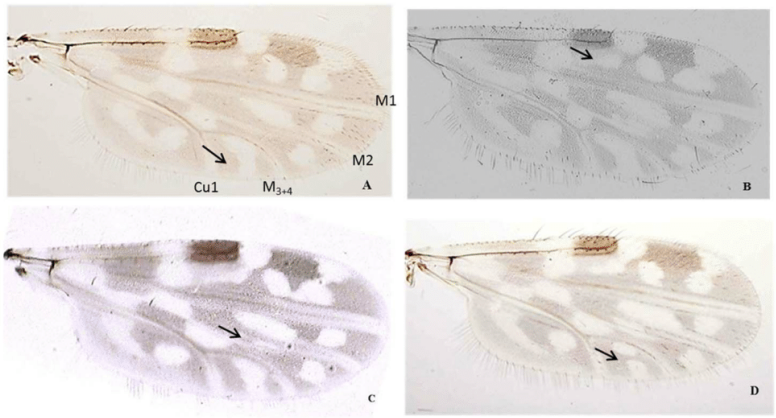

Figure 3. Variation in the wing patterns of *C. subschultzei* and *C. oxystoma* found in Senegal: *Culicoides subschultzei* typical form (A), *Culicoides oxystoma* typical form (B) and morphological variants: absence of pale spots in the second median cell (C) and presence of two pale spots in cubital cell (D).
doi:10.1371/journal.pone.0084316.g003

Table 2. Estimates of evolutionary divergence of sequence pairs between and within populations and species.

	Within species/group mean distance	*C. enderleini*	*C. kingi*	*C. nevilli*	*C. oxystoma* Australia	*C. oxystoma* Israel	*C. oxystoma* Japan	*C. oxystoma* Senegal
C. enderleini	0.022	–	–	–	–	–	–	–
C. kingi	0.015	0.0132	–	–	–	–	–	–
C. nevilli	0.028	0.12	0.106	–	–	–	–	–
C. oxystoma Australia	0.000	0.125	0.102	0.07	–	–	–	–
C. oxystoma Israel	0.015	0.131	0.103	0.079	0.019	–	–	–
C. oxystoma Japan	0.005	0.124	0.101	0.073	0.015	0.021	–	–
C. oxystoma Senegal	0.009	0.131	0.098	0.09	0.041	0.046	0.043	–
C. subschultzei	0.011	0.119	0.112	0.054	0.082	0.092	0.091	0.1

Analyses were conducted using the Jukes-Cantor model. The analysis involved 82 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 472 positions in the final dataset.

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T92+G+I and HKY+G. The best fit model (T92+G) was used for both ML and BA analysis. Summary sequence statistics were generated using MEGA v5. Bayesian analyses was performed using MrBayes [47] with the following settings: the ML model employed two substitution types ("nst = 2"), with rate variation across sites modeled using a gamma distribution (rates = "gamma"); Markov Chain Monte Carlo searches were done with four chains for 500,000 generations, with trees sampled every 100 generations (the first 1,000 trees were discarded as "burn in"). The appropriate burn-in fraction and convergence of the Markov Chain Monte Carlo chains were graphically assessed by evaluating the stationary phase of the chains using Tracer v1.5 [48]. Convergence metrics provided by MrBayes were checked to ensure that the maximum standard deviation of split frequencies of any of the runs was under 0.05 and that the potential scale reduction factor for all parameters approached 1.0. *Culicoides imicola* was used as an out-group. Estimates of average evolutionary divergence over sequence pairs within and between groups were made using the Maximum Composite Likelihood model for the COI sequences. The average genetic distances between the clades inferred by phylogenetic analyses were computed by Tamura-3 parameter or Jukes-Cantor model with the program Mega v5. The two models gave the same genetic distance matrix (data shown for JC model).

Results

Morphological Identification

Morphological examination of 82,506 individuals from the 5 study sites revealed the presence of four species belonging to the Schultzei group (Figure 2). These species were referable to *C. kingi* (both the Kenya and Senegal forms sensu Cornet & Bruhnes), *C. oxystoma* (showing large phenotypic variation) (Figure 3), *C. enderleini* and *C. nevilli*. Although *C. nevilli* was present at three sites, the other species were abundant and equally present on the five sampled sites.

Phylogenetic Analysis

A total of 63 COI sequences were obtained referable to six species including *C. imicola* used as out-group (Table 1). Twenty COI sequences were added to the dataset from the literature (Table 1). Sequencing of COI sequences of *C. schultzei* was not successful. Four individuals of each *C. kingi* form were obtained (Kenya form: KF682484–KF682485, KF682491–KF682492); Senegal form: KF682487, KF682489–KF682490, KF682494). The ML and BA trees depicted the same topology. Six distinct and well-supported phylogenetic lineages were clearly differentiated (Figure 4). One clade grouped the individuals identified as *C. kingi*, including both the Senegal and Kenya forms, and the three unidentified sequences from Israel (JN545050–51, JN545053). Another included all specimens of *C. enderleini* from all different location sites. Specimens of *C. oxystoma* formed two separate clades, one including all specimens from the Palaearctic and Australasian regions (Japan, Israel, and Australia) and the other including all individuals from Senegal. The mean genetic distance between these two clades was two times higher than the mean variation within a clade. The specimens from Senegal identified as *C. oxystoma* and representing all of the morphological variations illustrated in Figure 2 were clustered in the same clade. The maximum variation within a clade was 2.8% within the *C. nevilli* clade, and the minimum distance between clades being 1.5% between *C. oxystoma* from Australia and Japan (Table 2).

Discussion

At least four species belonging to the Schultzei group are now known from Senegal: *C. enderleini*, *C. kingi*, *C. nevilli*, and *C. oxystoma*. Our phylogenetic analyses have identified the three unidentified individuals (JN545050–51, JN545053) from Morag et al. (2012) as *C. kingi* which confirms the presence of this species in Israel. Our study confirmed that the Kenya and Senegal forms of this species, described by Cornet & Brunhes (1994) partially based on differing larval ecology, do not appear to be distinct species.

The relatively high genetic diversity observed within *C. nevilli* and *C. enderleini* probably reflects historically independent populations of these species on Madagascar and on mainland Africa as recently observed within Australian populations of *C. immaculatus* [49]. Overall, the *Culicoides* fauna of Madagascar has been understudied [39,50] and remains to be investigated.

The genetic similarity between Oriental and Australian specimens of *C. oxystoma* and those from Senegal confirms the identity of this species and is the first record of *C. oxystoma* in Africa south of Sahara. There has been a great deal of confusion in the literature between this species, *C. schultzei* and *C. subschultzei* [8,25,37]. Boorman (1989) suggested that most of the records of *C. schultzei* or the Schultzei group from north of the Sahara and eastwards through India refer to *C. oxystoma* and its presence in Senegal indicates that it is also present south of the Sahara. Taken

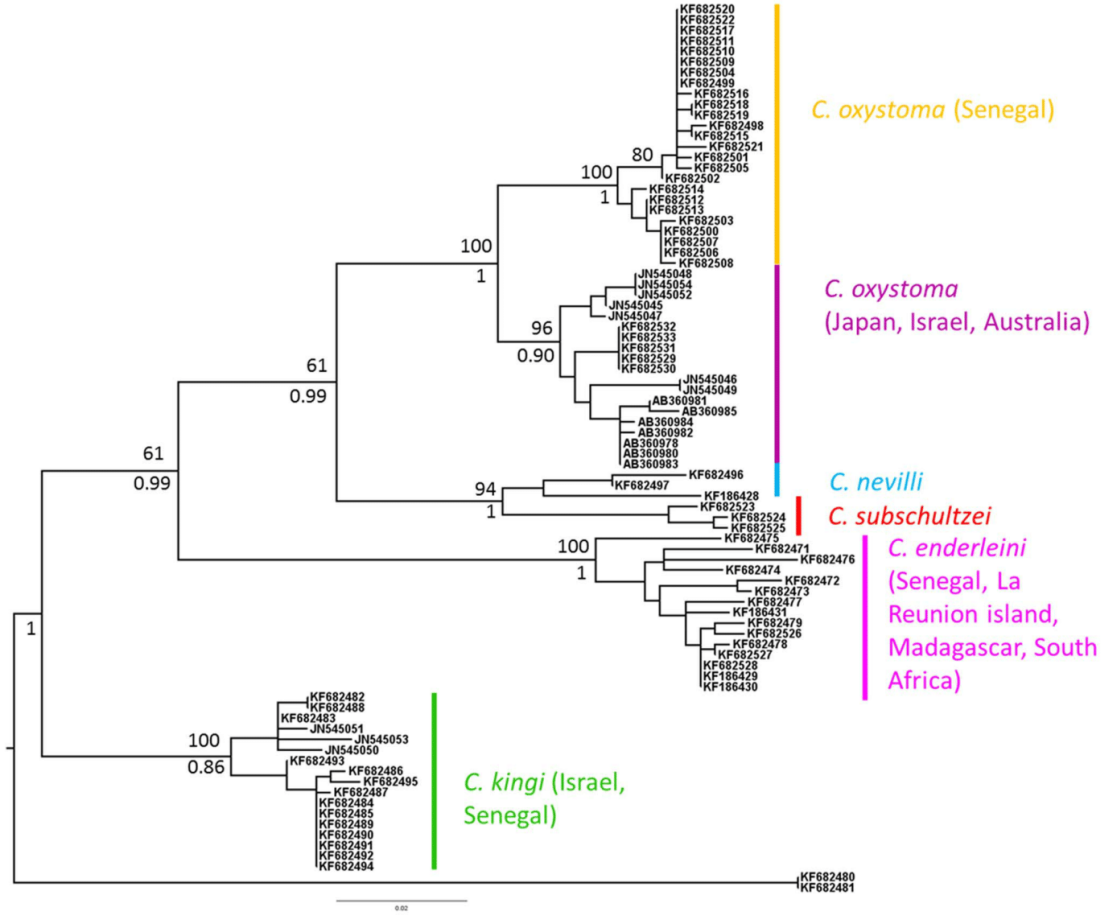

Figure 4. Phylogenetic analysis of species of the Schultzei group using COI sequence data. The topology shows Bayesian inference tree using program MrBayes. *Culicoides imicola* was designated as an outgroup taxon. Numbers indicate bootstrap values from ML (bottom) analyses, and posterior probabilities from Bayesian analysis (top). Only bootstrap values >60% and Bayesian posterior probabilities >0.5 are shown. Branch lengths represent nucleotide substitutions per site.
doi:10.1371/journal.pone.0084316.g004

together, Boorman et al (1989) and our results showed that multiple species of the Schultzei group are sympatric in the Middle East (for instance at least *C. kingi* and *C. oxystoma* in Israel), and we recommend use of the most updated keys (Boorman 1989; Cornet and Brunhes 1994) when identifying material collecting from this area.

Culicoides oxystoma is a widespread species with a wide phenotypic variation that warrants further exploration. Specimens exhibiting the full range of morphological variation observed in Senegal (Figure 3) were shown to be conspecific but also showed strong support for two lineages within *C. oxystoma* indicating some level of reproductive isolation within this species. This might confirm what Boorman suspected as a differentiation within *C. oxystoma*, creating an Afrotropical lineage and a Palearctic and oriental lineage [36]. Despite the morphological similarity between *C. oxystoma* and *C. subschultzei* [36], COI analysis indicates that these two species are distinct with *C. subschultzei* being more closely related to *C. nevilli* than to *C. oxystoma*. Further investigations need to be implemented including *C. schultzei*. Unfortunately, attempts to amplify DNA from the *C. schultzei* samples from South Africa were unsuccessful, probably because they were too old (collected in 1996). Further works will also need to include other known members of the group, *C. rhizophorensis* and *C. neoschultzei*, and probably look at other species showing morphological similarity [51].

Up to date no phylogenetic study has ever successfully validated the subgeneric classification of the genus *Culicoides* [4,5] and the validity of the Schultzei group, *C.* subg. *Remmia*, *C.* subg. *Oecacta*, and many other subgeneric groupings of *Culicoides*, remains doubtful. Augot et al. (2013) recently analysed the COI gene to test the monophyly of the Schultzei group and *C.* subg. *Oecacta* but their analysis were confounded by homoplasy of this gene and no conclusions could be drawn. They suggested the use of alternative genes to explore these relationships and it is likely that an integrated approach using a combination of morphological and molecular analyses might clarify this situation.

The wide distribution and economic importance of species of the Schultzei group highlight the need to re-evaluate the status of these species and a molecular approach might be an appropriate means of achieving that goal. Based on the numbers collected and wide distribution of the suspected vector species based on previous virus studies, these species can indeed play an important role in the epidemiology of AHSV in Senegal. In particular, future studies should focus on the differences between African and Oriental populations of *C. oxystoma* and establish the morphological and genetic limits of this species. Inclusion of material from the type locality of *C. oxystoma* in India would help to confirm the identity of specimens currently referred to this species.

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Author Contributions

Conceived and designed the experiments: MTB MF AGF MTS JB CG. Performed the experiments: MTB MF MB IM XA MD LG. Analyzed the data: MTB MF GAB YG KL GJV JCD CG. Contributed reagents/materials/analysis tools: GAB KL GJV JCD. Wrote the paper: MTB MF AGF GAB YG KL GJV MTS JB JCD TB CG.

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Title: Ecology and integrative taxonomy of biting midges of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) in the Afrotropical region

Key words: Systematics, taxonomy, bio-ecology *Culicoides*, Senegal, Afrotropical region

Summary:

In a context of emergence or re-emergence of vector-borne diseases, certain species of *Culicoides* (Diptera: Ceratopogonidae) are involved in the transmission of certain viruses (Reoviridae: *Orbivirus*) and nematodes (Onchocercidae: *Mansonella*) in the Afrotropical region. However, the systematic and taxonomic schemes as well as the bio-ecology of species of veterinary interest remain to be explored. This work of integrative taxonomy aims to achieve (i) a systematic and taxonomic revision of species belonging to subgenera and groups of veterinary interest using a multi-marker molecular phylogeny and species delineation, and (ii) to develop molecular tools for studying the bioecology of species of veterinary interest and dynamics of their immature populations.

Our results show (i) the presence of three monophyletic clades, the Imicola group, the Milnei group and the subgenus *Remmia*, (ii) a new species for science named *C. sp*. # 22 and affiliated into the subgenus *Avaritia*, Imicola group, (iii) the presence of a new undescribed species named *C. sp*. # 54 belonging to the Dasyops group, subgenus *Avaritia*, (iii) affiliating the Similis and Neavei species groups to the subgenus *Synhelea*, and (iv) cryptic species within *C. oxystoma* (subgenus *Remmia*). From a bioecological point of view, this work combining entomological follow-up and molecular identification with a library of barcode sequences allowed to describe the trophic behavior of *C. imicola*, *C. kingi* and *C. oxystoma* as well as their larval habitats in equine environments of the Niayes area in Senegal. This work completes the corpus of knowledge about the genus *Culicoides* in the Afrotropical region to improve our knowledge on the epidemiology of the transmitted pathogens and to propose research tracks to better control the immature and adult populations of the vector species in order to better anticipate and prevent *Culicoides*-borne diseases outbreaks.

Titre : Écologie et taxonomie intégrative des moucherons piqueurs du genre *Culicoides* Latreille (Diptera : Ceratopogonidae) en région Afrotropicale

Mots clés : Systématique, taxonomie, bio-écologie, *Culicoides*, Sénégal, région Afrotropicale

Résumé :

Dans un contexte d'émergence ou de réémergence des maladies à transmission vectorielle, certaines espèces de *Culicoides* (Diptera : Ceratopogonidae) sont impliquées dans la transmission de certains virus (Reoviridae : *Orbivirus*) et de nématodes (Onchocercidae : *Mansonella*) en région Afrotropicale. Cependant, le schéma systématique et taxonomique ainsi que la bio-écologie des espèces d'intérêt vétérinaire restent à explorer. Ce travail de taxonomie intégrative a pour objectifs de réaliser (i) une révision systématique et taxonomique des espèces appartenant à des sous-genres et des groupes d'intérêt à partir de phylogénie moléculaire multi-marqueur et de délimitation d'espèces, et (ii) de développer des outils moléculaires pour l'étude de la bio-écologie des espèces d'intérêt vétérinaire et de la dynamique de leurs populations d'immatures.

Nos résultats mettent en évidence (i) la présence de trois clades monophylétiques le groupe d'espèces Imicola, le groupe d'espèces Milnei et le sous-genre *Remmia*, (ii) une nouvelle espèce pour la science nommée *C. sp.* #22 et affiliée au sous-genre *Avaritia*, groupe Imicola, (iii) la présence d'une nouvelle espèce non-décrite nommée *C. sp.* #54 appartenant au groupe d'espèces Dasyops, sous-genre *Avaritia*, (iii) d'affilier les groupes d'espèces Similis et Neavei au sous-genre *Synhelea* et, (iv) de poser l'hypothèse d'une présence d'espèces cryptiques au sein de *C. oxystoma* (sous-genre *Remmia*). D'un point de vue bio-écologique, le travail réalisé combinant suivi entomologique et identification moléculaire avec une librairie de séquences barcodes a permis de décrire le comportement trophique de *C. imicola*, *C. kingi* et *C. oxystoma* ainsi que leurs habitats larvaires dans des environnements équins de la zone des Niayes au Sénégal. L'ensemble de ce travail permet de compléter le corpus de connaissances sur le genre *Culicoides* en région Afrotropicale afin d'améliorer la compréhension de l'épidémiologie des pathogènes transmis et proposer des pistes de recherches pour mieux contrôler les populations immatures et adultes des espèces vectrices afin de mieux anticiper et prévenir des évènements sanitaires.