**UAB**

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Facultat de Biociències

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Unitat de Zoologia

Tesi doctoral

## El gènere *Culicoides* a la Península Ibèrica: estudi de la biologia de potencials vectors dels virus de la Llengua Blava i Schmallenberg

Memòria de tesi doctoral presentada per Sandra Talavera Forcades per a optar al grau de Doctor en Biologia Animal sota la direcció del Dr. Nonito Pagès, i la tutorització del Dr. Fernando García del Pino.

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**Abstract**

## Abstract

Around 1,400 species of biting midges of the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae), have been described in the world, some of these are well known transmitters of protozoa, filarial worms and viruses that affect humans and domestic and/or wild animals. The Bluetongue virus (BTV) and the recently emerged Schmallenberg virus (SBV) are responsible of important infectious, non-contagious, insect-borne viral diseases found in domestic and wild ruminants and transmitted by *Culicoides* spp.

Two research axes are distinguished in the doctoral thesis to improve our comprehension on the epidemiology of Bluetongue (BT) and Schmallenberg (SB) diseases. The first research axe provides an improvement on the knowledge of the diversity, morphology and distribution of *Culicoides* species in the Iberian Peninsula. Data derived from morphology and sequencing allowed to detect the presence of 26 species not recorded previously in the surveillance area. Two eco-zones were detected in the region; the northern had species with typical northern Palaearctic European distribution whereas the southern one had species with typical southern Palaearctic distribution. A concise analysis of morphological traits and DNA sequencing was performed on specimens of the Pulicaris and Punctatus groups. Results revealed the presence of two new species in the subgenus *Culicoides*: *C. cryptipulicaris* and *C. quasipulicaris,* both species phylogenetically closely related to other *Culicoides* acting as diseases-vectors in Europe. The second research axe aimed at characterizing *Culicoides* midge communities in natural ecosystems inhabited by different wild ruminants along the Iberian Peninsula. The results showed that at a local scale, the presence of major BTV and SBV vector species in areas with wild ruminants was coincident with that of the nearest sentinel farms. Data suggested certain species could play a prominent role as bridge vectors for different pathogens between wild and domestic ruminants. Results support the hypothesis that wild ruminants act as reservoir for BT and SB, and could eventually be involved in the reintroduction of such diseases into livestock farms. In this context, it is essential to define precisely the bloodfeeding behaviour of *Culicoides* communities in contact with wild ruminants. Bloodmeal origin (host donor) was successfully determined in 114 out of 224 blood engorged females collected in natural ecosystems. The major BTV and SBV vector species were detected among the 14 *Culicoides* species with a recent bloodmeal. *Culicoides* fed more frequently on mammals (91.1%) than on birds (8.9%). Among hosts, red deer was the most frequent host biten (66.7%), followed by human (13%) and fallow deer (6.1%).

## Resum

Al voltant d'unes 1.400 espècies del gènere *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) s'han descrit a nivell mundial, algunes de les quals són conegudes com a transmissores de protozous, filàries i virus que poden afectar tant a humans com a animals domèstics i/o salvatges. El virus de la Llengua Blava i el recent descobert virus Schmallenberg són responsables de malalties infeccioses no contagioses, detectades en remugants domèstics i salvatges, transmeses per espècies del gènere *Culicoides*.

Al llarg d'aquesta tesi doctoral podem diferenciar dos eixos de recerca que ens permeten millorar en el coneixement de la epidemiologia de les malalties de la Llengua Blava (LB) i Schmallenberg (SB). El primer eix de recerca proporciona una millora en el coneixement de la diversitat, morfologia i distribució de les espècies de *Culicoides* a la Península Ibèrica. Les dades obtingudes a través de la morfologia i la seqüenciació del ADN ens han permès detectar la presència de 26 espècies no identificades prèviament a l'àrea d'estudi. En aquesta àrea dues zones ecològiques s'han pogut diferenciar, la zona del nord amb espècies amb distribució típica del nord paleàritc europeu, i la zona del sud amb espècies amb una distribució típica del sud paleàrtic. Un anàlisi concís de les característiques morfològiques i les seqüències d'ADN es va realitzar als individus pertanyents als grups Pulicaris i Punctatus. Els resultats obtinguts revelen la presència de dues noves espècies dins el subgènere *Culicoides*: *C. cryptipulicaris* i *C. quasipulicaris,* ambues espècies es troben estretament relacionades filogenèticament a altres espècies de *Culicoides* que actuen com a vectors de malalties a Europa. El segon eix de recerca està dirigit a caracteritzar les comunitats de *Culcioides* presents a diverses àrees naturals habitades per diferents espècies de remugants salvatges localitzades al llarg de la Península Ibèrica. Els resultats mostren que a escala local, la presència de les principals espècies vectores dels virus de la LB i SB en les àrees de remugants salvatges coincideix amb la de les granges sentinella properes. Les dades suggereixen que aquestes espècies podrien tenir un paper destacat com a vectors pont de diferents patògens entre remugants domèstics i salvatges. Els resultats donarien suport a la hipòtesi de que els remugants salvatges podrien actuar com a reservori de la LB i SB, i posteriorment estar involucrats en la reintroducció d'aquestes malalties a les granges de remugants domèstics. En aquest context, és essencial el coneixement precís dels hàbits alimentaris de les comunitats de *Culicoides* en contacte amb remugants salvatges. L'origen de la sang, es va poder determinar en 114 de les 224 femelles amb l'abdomen amb sang

capturades en els ecosistemes naturals, pertenyents a 14 espècies de *Culicoides* diferents. El 91.1% dels hostes escollits pels *Culicoides* van ser mamífers i el 8.9% aus. L'hoste més abundant va ser el cérvol (66.7%) seguit per l'home (13%) i la daina (6.1%).

**Introducció**

# Introducció

## El gènere *Culicoides*

### Generalitats

El gènere *Culicoides* Latreille, 1908 està format per uns petits dípters pertanyents a la família Ceratopogonidae, constituïda per aproximadament 125 gèneres i més de 5.500 espècies, de les quals només unes poques són hematòfagues de vertebrats i totes elles pertanyen a quatre gèneres: *Austroconops*, *Culicoides*, *Forcipomya* i *Leptoconops*. Són uns dels dípters hematòfags més petits i abundants del món, mesuren entre 1 i 3mm de longitud i tenen una distribució Holàrtica estant presents en totes les regions del món, des del nivell del mar fins els 4.000m d'altitud, exceptuant l'Antàrtida, Nova Zelanda i les Illes Hawaii (Mellor *et al*., 2000). Aproximadament unes 1.400 espècies de *Culicoides* han estat descrites i classificades en 39 subgèneres (Borkent i Wirth, 1997 actualitzat 2012). Es coneixen 130 espècies a Europa (Szadziewski i Borkent, 2004 actualitzat 2013; Ramilo *et al*., 2013) de les quals 81 s'han trobat a Espanya (Alarcón-Elbal i Lucientes, 2012). Un 96% d'aquestes espècies són hematòfagues obligades i tenen un ampli rang d'hostes, principalment mamífers i aus. El gènere *Culicoides* ha adquirit un paper molt rellevant en sanitat animal. Això es deu a que algunes espècies actuen com a transmissors de malalties, sent vectors de protozous, filàries y diferents virus, que poden afectar tant a humans com a animals domèstics i salvatges (Mellor *et al*., 2000).

### Biologia

Generalment les femelles de *Culicoides* posen els ous en zones amb humitat i riques en matèria orgànica, condicions indispensables pel posterior desenvolupament dels estadis immadurs (Kettle, 1962; Conte *et al*., 2007). Es coneixen molts tipus de microhàbitats utilitzats com a zones d'ovoposició com vores de llacs, rieres o rius, femtes de bestiar, acumulacions de fulles, restes vegetals i forats d'arbres entre d'altres (Uslu i Dik, 2010). El cicle biològic dels *Culicoides* inclou quatre estadis successius de desenvolupament (figura 1): ou, larva, pupa i adult.
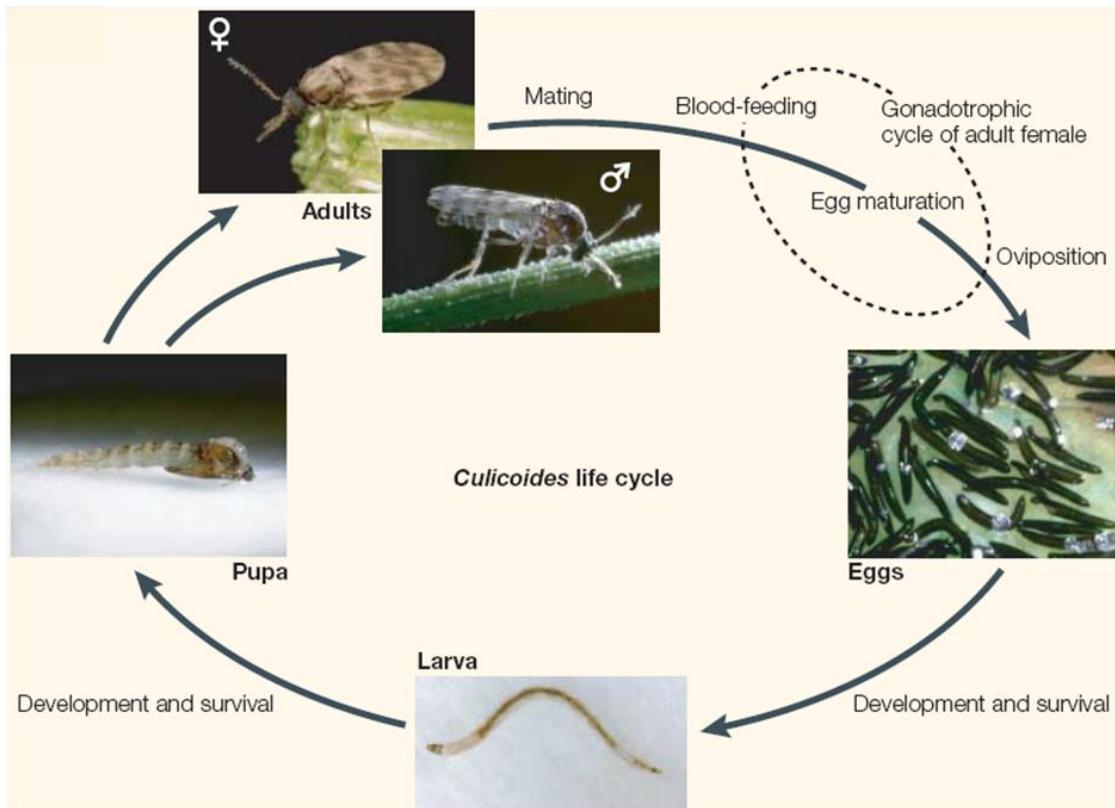
**Figura 1**. Cicle biològic dels *Culicoides* (Purse *et al.*, 2005)

**i) Ou:** són dipositats per les femelles a partir de les 48 hores i fins 2 setmanes després de la ingesta de sang depenent de la temperatura ambiental (Gerry i Mullens, 2000). Tenen forma allargada (400-500μ) i són de color clar al moment de la posta però es van enfosquint ràpidament amb el pas de les hores. El número d'ous per posta és molt variable podent oscil.lar entre 10 i 600, depenent de l'espècie (Blackwell i King, 1997) i altres condicions com la quantiat de sang ingerida per la femella. El temps d'eclosió és variable, en funció de l'espècie i les condicions climàtiques, havent-se observat períodes de 7-8 mesos per l'espècie *C. grisescens* (Parker, 1949) i períodes de 2-3 dies en el cas de *C. imicola.*

**ii) Larva:** les larves es desenvolupen a través de quatre estadis larvaris (L1, L2, L3 i L4) amb una duració variable regulada per la temperatura, el fotoperíode i la disponibilitat de nutrients. D'aquesta manera, podem trobar casos en que els quatre estadis larvaris es poden completar en tan sols una setmana, com és el cas de l'espècie tropical *C. loxodontis* (Meiswinkel, 1992). Per altra banda, arribem a trobar casos com el de les espècies àrtiques, en que poden tardar fins a dos anys (Downes, 1962). Les larves tenen una longitud aproximada de 0.5mm en estadi L1 i de 1cm en L4. Presenten una regió cefàlica ben esclerotitzada i no tenen potes ni pèls al llarg del

cos, fet que facilita el moviment ondulatori serpentejant que utilitzen per desplaçar-se a través del medi. Les larves es localitzen a les capes superficials del medi en el que viuen a profunditats de 0 a 12cm, tot i que la majoria es troben entre 0 i 5 cm i en molt poques ocasions es poden trobar a profunditats més grans de 8 cm (Uslu i Dik, 2006). D'aquesta manera, les condicions edàfiques del medi influeixen especialment en la composició de les poblacions presents en una zona (Foxi i Del Rio, 2010). Les larves presenten una alimentació molt variada segons el medi en el qual es desenvolupen. Es poden alimentar de detritus, bacteris, algues, fongs, protozous, rotífers, diatomees i inclús nematodes i petits invertebrats aquàtics i semiaquàtics entre altres organismes (Blanton i Wirth, 1979; Chaker, 1983). Des del punt de vista morfològic de les estructures encarregades de triturar l'aliment podem diferenciar espècies mastegadores i espècies xucladores-cribadores (Chaker, 1983).

**iii) Pupa:** generalment el més breu dels quatre estadis, durant el qual la pupa es manté en flotació en la superfície del medi. La pupa té un parell de protuberàncies respiratòries protoràciques que repel.leixen l'aigua que juntament a una butxaca d'aire situada sota els pegats alars o pteroteques faciliten la seva flotació mantenint-la en la superfície.

**iv) Adult o imago:** els mascles emergeixen abans que les femelles i la maduració de l'esperma es produeix en 24 hores. Les femelles a les 24 hores ja ingereixen sang i realitzen la còpula generalment en forma d'eixam (Roeder *et al*., 1991). La postura de la còpula consisteix generalment en la unió dels extrems del mascle i la femella, amb el cap mirant en sentits oposats. La genitalia del mascle rota 180º per posicionar-se en contacte amb la genitalia femenina. Després de la inseminació, ambdós sexes, especialment la femella, utilitzen les potes posteriors per separar-se (Blanton i Wirth, 1979). Tot i que la longevitat dels *Culicoides* en estat adult no ha estat ben estudiada, certs assajos de laboratori confirmen que poden arribar a viure fins a 90 dies (Nevill, 1971; Boorman, 1991). En condicions normals els adults poden volar activament com a molt uns pocs centenars de metres. Però en determinades condicions de temperatura del sòl, es formen corrents ascendents que poden elevar-los desenes de metres. Aleshores, si en aquell moment es generen corrents d'aire amb una velocitat aproximada de 10m/s, la temperatura no sobrepassa els 30ºC i la humitat és superior al 25%, poden ser transportats centenars de kilòmetres (Ducheyne *et al*., 2007; Lucientes *et al*., 2008).

**Alimentació, especificitat d'hoste**

La majoria dels *Culicoides* tenen activitat crepuscular, centrant la seva activitat entre la posta de sol i l'albada (Mellor *et al.*, 2000). En aquest moment aprofiten que disminueix la temperatura i augmenta la humitat ambiental, tot i que algunes espècies estan actives i poden picar durant el dia (Balenghien *et al*., 2008). La majoria de femelles en estat adult s'alimenten de sang per obtenir un aport extra de proteïna i així poder realitzar la síntesi dels ous. No obstant hi ha algunes espècies autògenes com *C. impunctatus* i *C. circumscriptus*, que tenen la capacitat de fer una primera posta d'ous sense la necessitat d'una font proteica exògena (Borkent i Spinelli, 2007). Tanmateix femelles i mascles també es solen alimentar de fluids vegetals rics en sucres i aigua per l'obtenció d'energia.

Les femelles de *Culicoides* s'alimenten d'una gran varietat d'hostes que detecten mitjançant diferents estímuls de naturalesa visual i química (Van Middelaar, 2008; De Jong i Knols, 1996). Existeixen multitud de substàncies emeses pels hostes vertebrats resultat del seu metabolisme com el $CO_2$, l'àcid làctic, acetona o octenol que són estimulants de certs receptors d'un ampli ventall d'insectes hematòfags (Bhasin *et al*., 2000). També trobem altres factors ambientals com la radiació solar, la temperatura, la humitat i la velocitat del vent que poden condicionar la seva activitat (Blackwell *et al*., 1996; Blackwell *et al*., 1997). Els *Culicoides* tenen un sistema olfactiu sensible constituït per les antenes i els palps maxil.lars, que en combinació amb altres sentits els hi serveixen per localitzar els hostes amb elevada precisió (Blackwell, 2008). Un cop els insectes han aterrat sobre l'hoste, estímuls de contacte com la temperatura i la humitat corporals seran determinants en la decisió de picar o no (Torres-Estrada *et al*., 2003). L'estudi de les preferències alimentàries dels insectes hematòfags és clau per entendre l'epidemilogia de les diferents malalties vectorials que transmeten (Ninio *et al*., 2011).

El cicle gonotròfic es defineix com el període de temps entre una alimentació de sang completa per part de la femella i la següent, amb la conseqüent maduració i ovoposició dels ous. Majoritàriament s'accepta que els diferents estadis del cicle es poden diferenciar amb l'observació de l'abdomen (Dyce, 1969), tot i que alguns autors han demostrat les limitacions del mètode (Braverman i Mumcuoglu, 2009). Podem diferenciar quatre estadis gonotròfics (figura 2):

**i) Femelles nul.lípares:** no s'observa pigmentació abdominal, l'abdomen és d'un color blanc-transparent indicant que la femella encara no s'ha alimentat de sang.

**ii) Femelles alimentades amb sang no digerida:** s'observa l'abdomen inflat i de color vermellós degut a la sang ingerida recentment.

**iii) Femelles gràvides:** s'observa l'abdomen ple d'ous preparats per la seva ovoposició.

**iv) Femelles pares:** s'observa l'abdomen amb una pigmentació granatosa degut a les restes de sang ingerida, els ous ja han estat dipositats.



**Figura 2**. Estadis gonotròfics. D'esquerra a dreta: ♀ nul.lípara, ♀ para, ♀ gràvida i ♀ alimentada amb sang (Foto: David Borràs, IBABSA)

### Morfologia de l'adult, clau per la identificació morfològica

A nivell taxonòmic, el gènere *Culicoides* resulta un grup complex. Al llarg dels anys s'ha anat veient les dificultats taxonòmiques que aquest gènere presenta degut a que existeixen espècies filogenèticament molt properes que resulten molt difícils d'identificar morfològicament (Campbell i Pelham-Clinton, 1960). Tot i això la morfologia clàssica ens permet la identificació de la majoria d'espècies i gràcies a eines recents com la biologia molecular i la morfometria geomètrica actualment també és possible identificar aquelles espècies críptiques (Meiswinkel *et al.*, 2004; Gomulski *et al.*, 2006; Pagès *et al.*, 2009; Muñoz-Muñoz *et al.*, 2011; Lassen *et al.*, 2011; Muñoz-Muñoz *et al.*, 2014; Sarvasova *et al.*, 2014; Nielsen i Kristensen 2015).

Els adults són insectes de cos compacte amb dos ales que es situen en forma de teulada sobre l'abdomen (figura 3) quan l'insecte està en repòs, podem diferenciar-ne les següents parts:

**i) Cap:** de forma arrodonida, lleugerament aplanat en sentit antero-posterior, ocupat en gran part per dos ulls compostos. Davant dels ulls lateralment s'inserten les antenes, els palps i les peces bucals. Estructures claus per la determinació morfològica de les espècies (figura 4a).

Antenes: presenten dimorfisme sexual sent filiformes les de les femelles i plumoses les dels mascles (figura 4b). Formades per quinze artells en els quals trobem diferents òrgans sensorials entre els que destaquen les sensiles coelocòniques que són petites fossetes sensorials (figura 4c). El nombre i distribució d'aquestes sensiles és característic de cada espècie, resultant un caràcter taxonòmic molt important. Palps maxil.lars: estructures sensorials situades externament al costat de les peces bucals. Formats per 5 artells, els dos primers solen estar semi-soldats i el tercer sol estar engruixit i posseeix unes agrupacions de sensiles que actuen com a òrgans sensorials al tenir un gran nombre de receptors, conegudes amb el nom de fosseta sensorial, estructura també clau en la determinació morfològica de les diferents espècies (figura 4d).

Peces bucals (figura 4e): de tipus tallador-xuclador, consten d'una probòscide, un parell de mandíbules serrades amb dents en la seva part distal interna, un parell de maxil.les i la hipofaringe. Els mascles tenen les peces bucals més reduïdes degut als seus hàbits alimentaris florícoles.



**Figura 3**. Adult (♀) del gènere *Culicoides*
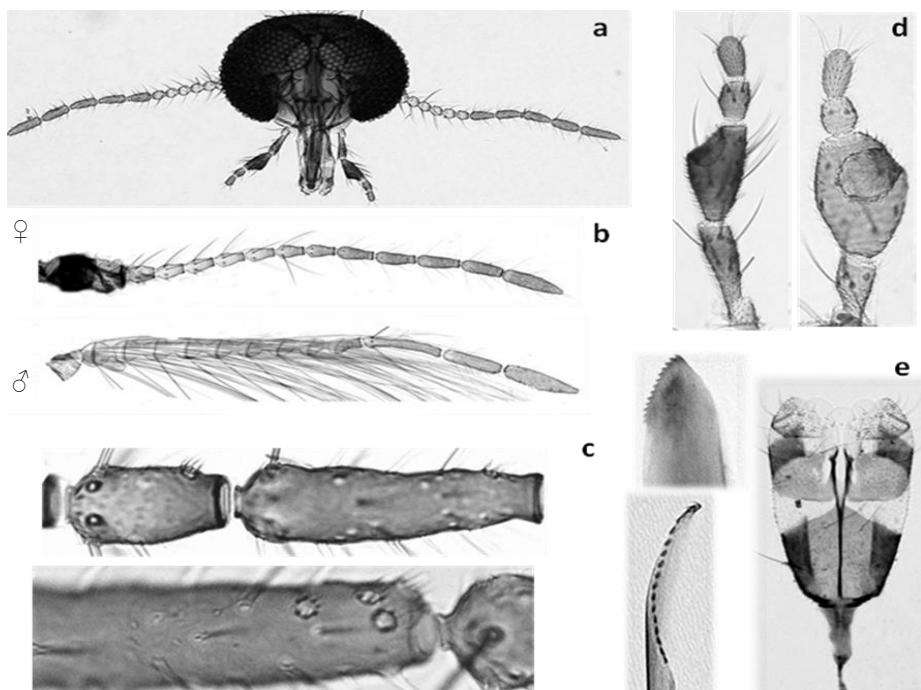
**Figura 4.** a, cap; b, antena; c, sensiles coelocòniques; d, palp maxil.lar; e, peces bucals

**ii) Tórax:** on s'inserten els apèndixs locomotors, un parell d'ales i tres parells de potes.

Ales: són membranoses i grans en relació al tamany del cos de l'insecte, tenen una venació característica que permet diferenciar-les de la resta de ceratopogònids (figura 5). Estan recobertes per microtriques més o menys pigmentades que defineixen diferents patrons de taques característics de gran part de les espècies de *Culicoides* (Boorman, 1988; Meiswinkel *et al.*, 2004), que permeten la determinació macroscòpica de moltes espècies (figura 6).

Potes: el parell de potes anterior destaca per la presència d'una espina apical tibial, el parell del potes del mig no posseeix cap estructura característica i el posterior té una pinta d'espines en la part apical de les tibies, en el 5è tarsòmer té un parell d'ungles corves i un empodi rudimentari.
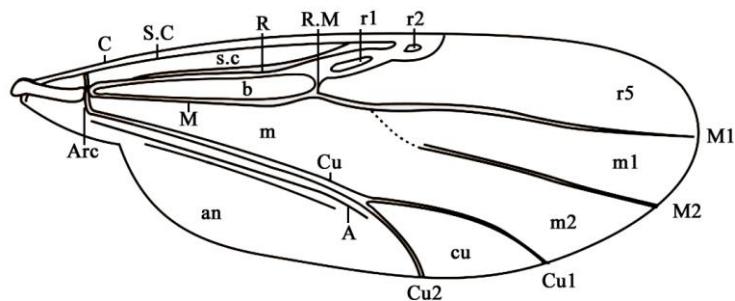
**Figura 5.** Esquema de les diferents regions d'una ala de *Culicoides.* Venacions: C. costa; S.C. sub-costal; R. radial; M,M1,M2. mitjana i branques; Cu,Cu1,Cu2. cubital i branques; A. anal; R-M. radiomedial; Arc. àrculus. Cel.les: b. basal; s.c. sub-costal; r1. primera radial; r2. segona radial; r5. cinquena radial; m,m1,m2. mitjanes; cu. cubital; an. anal
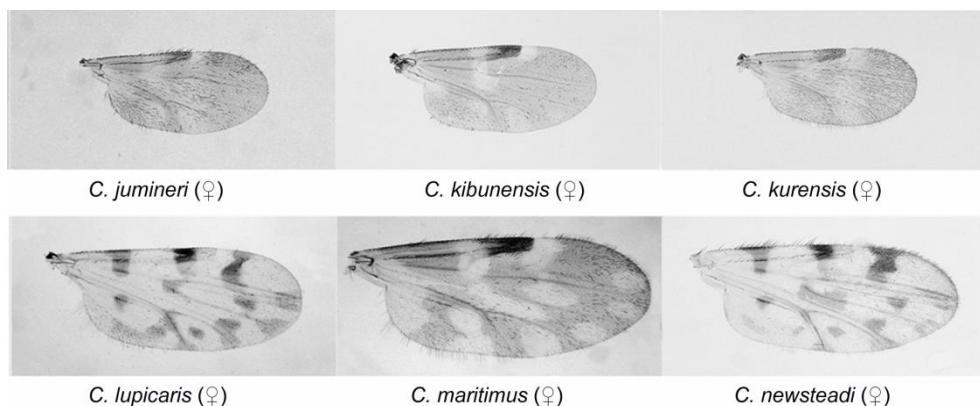


**Figura 6.** Patrons alars característics d'algunes espècies de *Culicoides*

**iii) Abdomen:** la femella té un abdomen relativament gruixut i a l'interior presenta unes estructures esclerotitzades denominades espermateques. Les espermateques generalment tenen forma oval o piriforme i estan unides per conductes hialins que es fusionen en un conducte comú, on es forma un petit anell esclerotitzat. La majoria d'espècies tenen dos espermateques funcionals i una tercera més petita rudimentària. El número i forma de les espermateques funcionals són importants per la identificació de les espècies (figura 7a). L'abdomen dels mascles és més allargat i acaba en una prominent genitalia que és de gran importància en la classificació de les espècies (figura 7b).
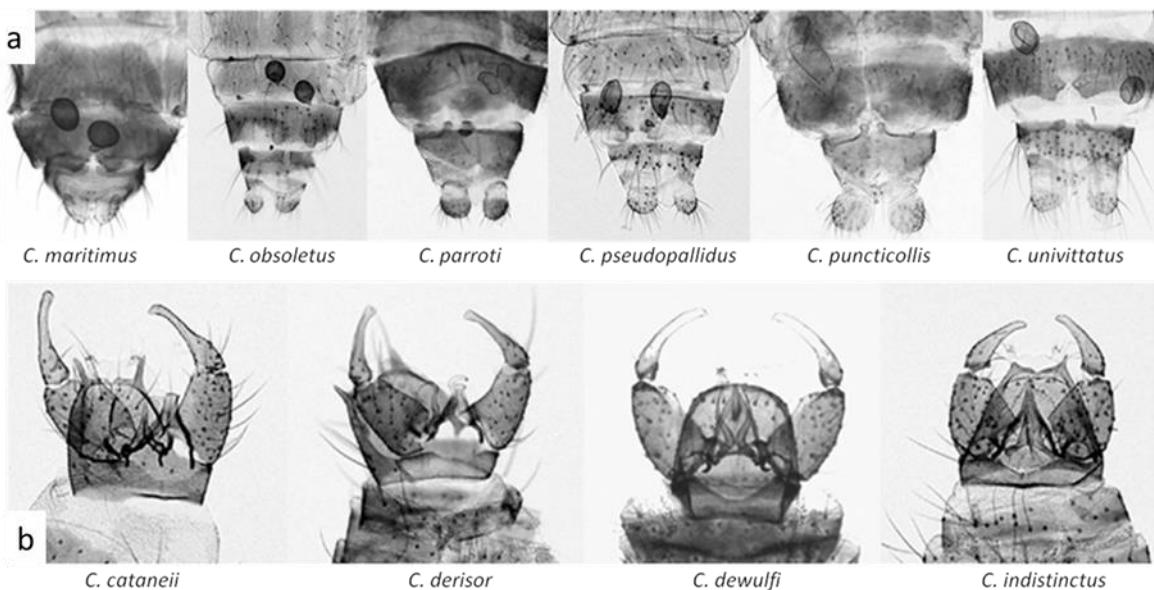
**Figura 7.** a. espermateques femenines de diferents espècies de *Culicoides*; b. genitalies masculines de diferents espècies de *Culicodies*

**El gènere *Culicoides* com a vectors de malalties**

Desde els anys 50 els *Culicoides* han estat coneguts per produir grans molèsties a nivell mundial a humans i bestiar a través de la seva picada. Els *Culicoides* han arribat a provocar importants pèrdues econòmiques afectant principalment el turisme de zones costaneres com les de Florida (Cilek *et al*., 2003) o Escòcia durant la temporada d'estiu (Mands *et al*., 2004; Carpenter *et al*., 2006). No ha estat fins el últims anys quan els *Culicoides* han adquirit gran importància com a transmissors de diferents malalties, principalment víriques (arbovirosis). Tot i això, també poden transmetre altres tipus de patògens com protozous i filàries, tant a humans com a animals domèstics i/o salvatges (Mellor *et al*., 2000).

Tot i que una gran quantitat d'artròpodes són capaços d'infectar-se amb un determinat patogen, només aquells que són capaços de mantenir el cicle del patogen en la natura es poden considerar vectors primaris de la malaltia. Un vector primari ha de cumplir una sèrie de requisits: i) al camp s'ha de trobar periòdicament infectat de manera natural, ii) el vector s'ha de poder infectar quan s'alimenti d'un hoste virèmic, iii) l'habilitat d'un vector infectat per transmetre el patogen s'ha de poder confirmar en condicions controlades i, iv) en condicions naturals el vector ha d'estar en contacte i alimentar-se de l'hoste vertebrat. Les espècies que compleixen aquests criteris es poden considerar potencials vectors primaris. Per altra banda, les espècies que només

compleixen algun d'aquests criteris s'han de considerar "possibles vectors" (WHO, 1967).

El conjunt de factors que fan d'un artòpode un bon vector, amb habilitat per infectar-se i transmetre un patogen, formen part d'un índex que anomenem capacitat vectorial. La capacitat vectorial d'un vector depèn de factors extrínsecs i de factors intrínsecs al vector. Entre els factors extrínsecs o ecològics trobem l´abundància del vector, la probabilitat de supervivència, el nombre de cicles gonotròfics al llarg de la seva vida, característiques del comportament alimentari, i com a factors intrínsecs aspectes genètics del vector. La competència vectorial es defineix com l'habilitat intrínseca del vector a infectar-se i transmetre un patogen (Higgs *et al*., 2005) que depèn de la genètica del vector i d'influències ambientals externes. Quan el *Culicoides* s'alimenta d'un hoste virèmic i ingereix la sang, el virus ha de ser disseminat a l'interior del vector. Per disseminar-se, ha de superar una sèrie de barreres fins arribar a les glàndules salivals, on es produirà la seva replicació i posterior alliberament a la saliva (Fu *et al*., 1999; Mellor, 2000; Mellor, 2004) (figura 8). Aquesta habilitat intrínseca del vector està determinada principalment per factors genètics.
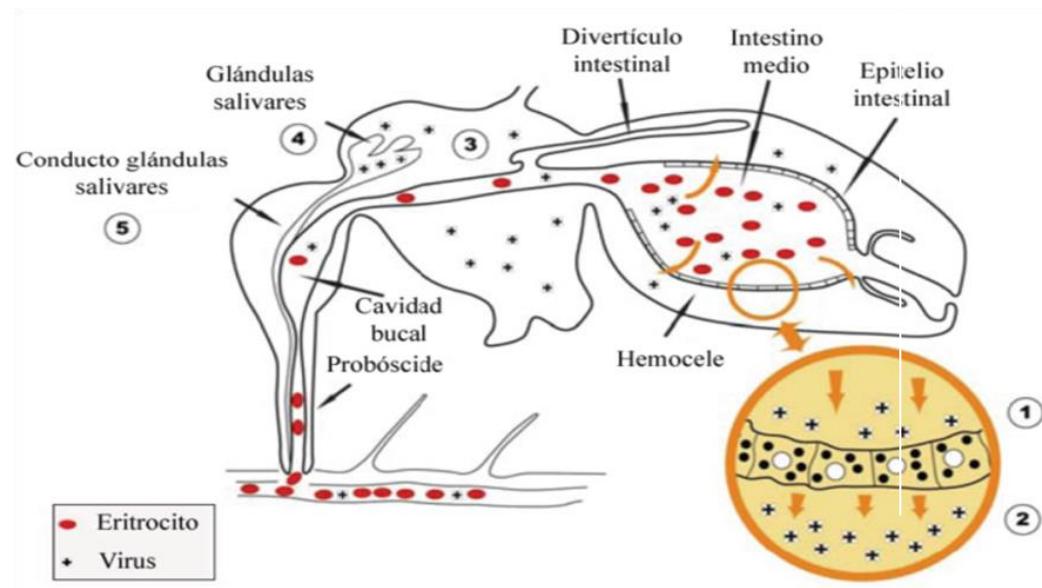


**Figura 8.** Disseminació vírica a l'interior de vectors competents

Podem considerar doncs que la capacitat vectorial engloba tant la interacció del vector amb el patogen (competència vectorial), com la interacció del vector amb l'hoste (Beaty i Marquardt, 1996).

La major repercussió dels *Culicoides* com a transmissors de malalties és en el camp de la sanitat animal. Els *Culicoides* són vectors, entre altres, dels virus de la LB (VLB), SB (VSB), pesta equina Africana (VPEA) la malaltia epizoòtica hemorràgica dels cérvols (VMEH), encefalitis equina (VEE), Akabane (VAKA), febre efímera bovina (VFEB) i Palyam.

Els *Culicoide*s també incideixen en el camp de la salut pública, on transmeten principalment dues malalties en latituds tropical i subtropical. El primer patogen és el virus d'Oropuche, responsable d'una de les arbovirosis més importants d'Amèrica del Sud i Amèrica Central, causant de múltiples epidèmies i milers de casos clínics a Brasil, Equador, Panamà i Perú (Pinheiro, 1981; Wats, 1997). El segon patogen són les filàries que provoquen la Mansonelliasis a Àfrica i Amèrica.

Els arbovirus es mantenen en la natura en cicles que impliquen la infecció alterna en hostes vertebrats i invertebrats. El cicle de transmissió de les arbovirosis transmeses per *Culicoides* s'inicia amb la picada d'una femella de *Culicoides* no infectada a un hoste virèmic. El *Culicoides* ingereix sang amb suficients partícules víriques com per poder-se infectar. A l'interior del *Culicoides* el virus necessita d'un període de temps (període d'incubació extrínsec) per disseminar, replicar en quantitat suficient i ser alliberat a la saliva per poder ser transmès. La durada del període d'incubació extrínsec és variable en funció del virus, les condicions ambientals i l'espècie de *Culicoides.* A partir d'aquest moment la femella de *Culicoides* infectada s'alimenta d'un hoste no infectat i l'infecta, sempre que l'hoste sigui susceptible a la malaltia. Després d'un període de latència o període d'incubació intrínsec, variable en funció del virus, condicions ambientals i espècie de vertebrat, l'hoste recent infectat desenvoluparà una virèmia. Si la virèmia de l'hoste és suficientment alta podrà infectar noves femelles de *Culicoides* que s'alimentin d'ell, tancant així el cicle de transmissió (figura 9).
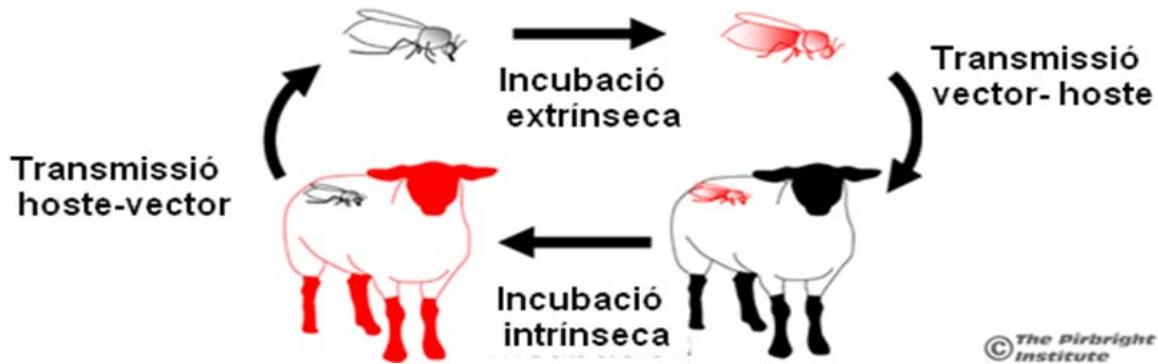
**Figura 9.** Cicle de transmissió d'arbovirosis (adaptat de "The Pirbright Institute")

**El virus de la Llengua Blava (VLB)**

La LB (febre catarral ovina o *Bluetongue*) és una malaltia vírica infecciosa no contagiosa. La malaltia és causada per un virus ARN de la família *Reoviridae* i gènere *Orbivirus* transmesa per vectors, que pot afectar tant a remugants domèstics com salvatges (MacLachlan, 2004; Stallnecht i Howerth, 2004).

La  malaltia va ser descrita per primera vegada a Sud-Àfrica l'any 1881 com a "febre catarral malàrica" (Spreull, 1905; Mehlhorn *et al*., 2007). L'any 1902, Spreull va detallar el quadre clínic associat a la malaltia (Gür *et al.*, 2008) i l'any 1906 Theiler va suggerir que l'agent etiològic causant de la LB era un virus. El nom de Llengua Blava prové de la paraula *bloutong*, amb la que els grangers de l'Àfrica austral describien la característica llengua blava que presenten  els remugants greument afectats per la malaltia.

Històricament aquesta havia estat una malaltia que es retringia a zones tropicals i temperades, on la distribució global era coincident amb la distribució dels vectors competents. Al voltant del món s'han identificat un total de 27 serotips fins al moment (Jenckel *et al*., 2015), molt heterogenis entre ells pel que fa a simptomatologia clínica, lesions, morbilitat i mortalitat en hostes susceptibles (MacLachlan *et al*., 2009).Tot i que existeixen vacunes eficients per immunitzar els hostes enfront la LB, aquestes solen ser específiques de serotip. Aquest fet complica les campanyes de vacunació ja que no existeix una protecció creuada. Per aquest motiu, certs autors recomanen tractar cada serotip de LB com una malaltia diferent (Lucientes,

2008). La LB és una malaltia que causa importants pèrdues econòmiques en bestiar domèstic. Les estimacions de les pèrdues econòmiques a nivell mundial produïdes pel VLB a finals del segle XX, han estat estimades en tres bilions de dòlars anuals (Tabachnick *et al.*, 1996).

La malaltia produeix generalment una simptomatologia clínica caracteritzada per presència de febre, hipersalivació, hiperlacrimació, descàrrega nasal, ulceració de les mucoses del tracte gastrointestinal superior entre altres. Aquests símptomes solen ser característics en bestiar oví i certs remugants salvatges (Taylor, 1986), tot i que també pot afectar altres espècies com camèlids i carnívors (OIE, 2009). La infecció pel VLB sol ser asimptomàtica en bestiar cabrú, boví i la majoria de remugants salvatges, afavorint així la presència no detectable d'animals que actuen com a resevori de la malaltia.

Es coneixen més de 1.400 espècies de *Culicoides* distribuïdes per tot el món. Tot i que el 96% d'aquestes són hematòfagues, fins al moment només s'han involucrat 32 espècies de *Culicoides* en la transmissió de la LB (Borkent, 2012; Meiswinkel *et al.*, 2004). Fa més de mig segle que els *Culicoides* van ser descrits com a vectors de la LB en condicions naturals (Du Toit, 1944). Al llarg dels últims anys, diverses espècies de *Culicoides* han estat proposades com a vectors en diferents brots a Europa, a través d'aïllaments virals en *Culicoides* capturats durant els brots de LB (Caracappa *et al.*, 2003; De Liberato *et al.*, 2005; Savini *et al.*, 2005), de detecció vírica mitjanant RT-PCR (Carcappa *et al.*, 2003; Meiswinkel *et al.*, 2007; Dijkstra *et al.*, 2008), i assajos d'infecció experimental (Carpenter *et al.*, 2006; Carpenter *et al.*, 2008).

Marcades diferències geogràfiques en quant a serotips de LB, tipus de bestiar afectat i presència d'espècies de *Culicoides* que actuen com a vectors són presents a nivell mundial (Gibbs i Greiner, 1994; Pritchard *et al.*, 2004; Tabachnick, 2004; Maclachlan i Osburn, 2006; Balasuriya *et al.*, 2008). Sent les espècies *C. imicola* i *C. bolitinos* les principals vectores de la malaltia a l'Àfrica subsahariana. *C. imicola* és probablement el vector més important al llarg de la conca Mediterrània, així com probablement en el Pròxim Orient. Les espècies *C. obsoletus, C. scoticus, C. chiopterus, C. dewulfi* i *C. pulicaris* a Europa (Meiswinkel *et al.*, 2007; Melhorn *et al.*, 2007; Dijkstra *et al.*, 2008; Goffredo *et al.*, 2015). A Sud Amèrica i Amèrica Central trobem *C. insignis* i *C. pusillus* (Mo *et al.*, 1994; Lager, 2004), mentre que *C. sonorensis* és el responsable de la transmissió de la LB a Amèrica del Nord excepte la zona sud-est dels EE.UU on *C. insignis* presenta el seu rang de distribució més septentrional (Tabachnick, 1996; Tabachnick, 2004).

Finalment, *C. brevitarsis* i *C. wadai* són els vectors primaris de la LB a Austràlia i el sud-est asiàtic (Melville, 2004; figura 10).
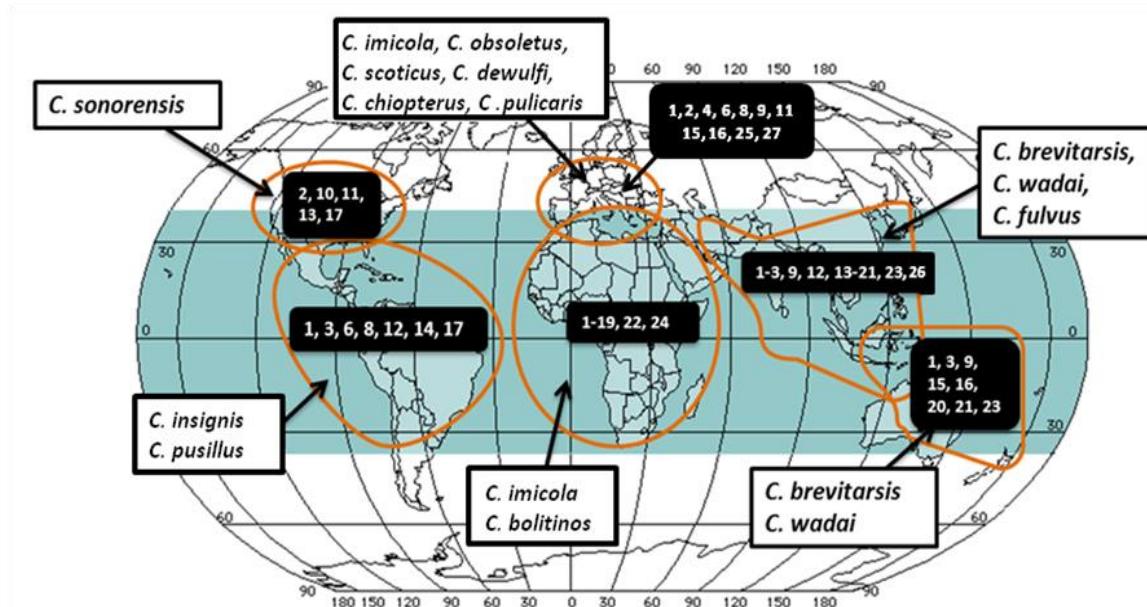


**Figura 10**. Distribució mundial dels diferents serotips de la LB i les diferents espècies de *Culicoides* implicades en la seva transmissió

Generalment es considera que la distribució i el manteniment de la LB, així com totes les arbovirosis, estan associats a unes condicions climàtiques apropiades, la presència d' hostes susceptibles i vectors competents en una mateix moment i lloc (Erasmus i Potgieter, 2009). Històricament, fins a finals del segle XX la LB es trobava delimitada entre les latituds 40ºN i 35ºS, en les zones tropicals i temperades de gran part del món afectant a zones del continent africà, Àsia i Amèrica amb escasses incursions a la conca Mediterrània (Roy, 1992; Purse *et al*., 2005). No obstant, en els últims 15 anys la seva distribució ha variat dràsticament a nivell mundial (Wilson i Mellor 2008; Purse *et al*., 2008). S'ha proposat que aquest canvi ha estat produït en gran part pel canvi climàtic, (Wittman *et al.,* 2001; Purse *et al.,* 2005). L'augment de les temperatures hauria provocat un canvi en la distribució i capacitat vectorial de les espècies de *Culicoides,* (Gerry *et al*., 2001; Purse *et al*., 2005; Purse *et al*., 2008; Wilson i Mellor, 2008; Gould i Higgs, 2009), afavorint la seva adaptació a zones tradicionalment més fredes (Purse *et al*., 2005; Hoffmannn *et al*., 2008; Rasmussen *et al*., 2010). Això hauria permès la disseminació de la malaltia fins els paral.lels 53ºN i 34ºS (MAGRAMA, 2013; figura 11).
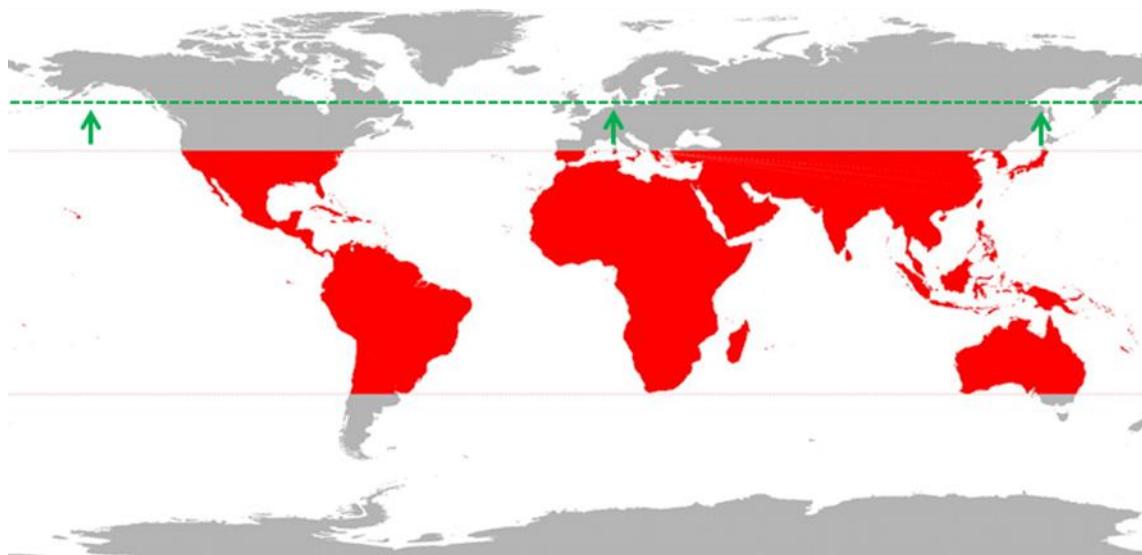
**Figura 11.** Rang de distribució de la LB (■ històric, ■ actual)

Existeixen diferents vies d'introducció de la LB en una zona geogràfica lliure de la malaltia: i) dispersió de vectors infectats a través del vent, els *Culicoides* poden ser transportats com plàncton aeri uns 700km a velocitats de 10-40Km/h, a 1,5Km sobre el nivell del mar en condicions climàtiques excepcionalment favorables (Sellers, 1992; Alba *et al.*, 2004), ii) trasllat passiu del vector infectat mitjançant transport marítim o terrestre (d'animals o plantes) podent ser transportats llargues distàncies entre diferents continents, tal i com va ocórrer a la Xina (Nie *et al.*, 2005). Les probabilitats d' introducció del VLB a través d'aquesta via són molt baixes (Napp *et al.*, 2009) degut a les diferències de temperatures ambientals entre les diferents zones del món, iii) moviments d'animals vius infectats i asimptomàtics (Alexander *et al.*, 1994; Alexander *et al.*, 1996; Jauniaux *et al.*, 2008), tot i que aquests moviments de zones endèmiques o epidèmiques cap a zones lliures de la LB estan fortament regulats per la legislació internacional, i iv) a través de vacunes vives atenuades (Sánchez Matamoros *et al.*, 2007).

Els primers brots de la LB es van donar a l'Àfrica l'any 1924, on probablement la infecció ja era endèmica en els remugants salvatges, i posteriorment a Chipre el 1943, estenent-se a Turquia i l'Orient mitjà l'any 1951 (Shimshony, 2004). Als EE.UU els primers registres de la malaltia daten dels anys 50 igual que a Europa quan durant aquests anys la LB va aparèixer a diferents països de la conca Mediterrània, arribant per primer cop al sud de la Península Ibèrica l'any 1956 (López i Sánchez-Botija, 1958). Al 1961 es va descriure la malaltia per primer cop al continent asiàtic (India), estenent-se posteriorment a altres països del continent com Xina,

Malàisia e Indonesia (Sapre, 1964). El primer cas a Austràlia data del 1975 (St George *et al*., 1978).

Europa ha estat tradicionalment una zona lliure de la malaltia de la LB, havent aparegut tan sols de manera esporàdica, sense que s'hagi arribat a establir (Mellor i Boorman, 1995). Al llarg dels anys podem remarcar dos esdeveniments importants, el primer va ser a partir de l'any 1998 quan es va detectar el serotip 9 a les Illes Gregues (Mellor i Wittmann, 2002), després la malaltia es va anar expandint cap al nord i l'oest amb l'aparició dels serotips 1, 4 i 16 (Mellor i Wittmann, 2002; Panagiotatos, 2004).També es va detectar el serotip 2 al nord d'Àfrica (OIE, 2000a i 2000b) i a diverses illes del centre i oest mediterrani i a Itàlia. L'any 2003 es va detectar una altra soca del serotip 4 a Sardenya i les Illes Balears que posteriorment es va expandir fins la Península Ibèrica i Còrsega. L'any 2006 es va detectar el serotip 1 a Espanya i Sardenya provinent del nord d'Àfrica. La majoria d'aquests brots de la malaltia es van associar a l'espècie *C. imicola* augmentant així el seu rang de distribució conegut fins el moment. El segon esdeveniment important es va produir l'agost del 2006 quan es va declarar el primer cas de LB al nord d'Europa, a latituds més elevedes del paral.lel 45º N, causat pel serotip 8 a Holanda. Posteriorment la malaltia es va estendre a  altres països com Bèlgica, Alemanya, Luxemburg, França, Dinamarca, Regne Unit, Suïssa, la República Txeca, Suècia, Hongria, Àustria i Itàlia (OIE, 2006a, 2006b, 2006c, 2006d i 2006e); l'origen i la ruta d'introducció d'aquest nou serotip exòtic de LB al nord d'Europa segueixen sent incerts. L'any 2008 Alemanya i Holanda van detectar circulació del serotip 6 sense observar simptomatologia clínica associada. L'anàlisi genètic dels aïllats vírics va mostrar una gran similitud amb la soca vacunal sud-africana modificada per ser utilitzada com a vacuna viva (ProMED-mail, 2009). A Bèlgica, es va detectar la circulació del serotip 11 no virulent amb gran similitud amb la soca vacunal sud-africana de referència (ProMED-mail, 2009). El serotip  VLB-25 es va aïllar l'any 2008 en mostres de cabres procedents de diferents països del centre d'Europa on no havia causat símptomes clínics (Chaignat *et al*., 2009; Chaignat *et al*., 2010). Anàlisis realizades a mostres d'arxiu demostren que el virus estava present a la regió desde l'any 1998 (Chaignat *et al*., 2010). Tot i això no s'ha trobat cap espècie de *Culicoides* amb restes del serotip del virus pel que no es descarta una transmissió transplacentària (Worwa *et al*., 2009; De Clercq *et al*., 2008). El serotip VLB-27 es va detectar a principis del 2014 en cabres sense símptomes clínics durant un programa de vacunació a Còrsega, l'origen del virus avui dia es desconeix (Jenkel *et al*., 2015).

**Pla de vigilància nacional**

En els últims quinze anys, fins a quatre serotips diferents del VLB s'han detectat a Espanya (1, 2, 4 i 8) (RASVE, 2013; Gómez i Tejedor, 2004). Als anys 2002 i 2003 es van donar alguns casos a les Illes Balears del serotip 2 i 4 respectivament, després de diferents estudis es va concloure que la via d'entrada de la malaltia a les Illes Baleras hauria estat el transport de *Culicoides* infectats a través de corrents aeris des d'àrees properes afectades pel mateix serotip, probablement Sardenya (Alba *et al.,* 2004). Arrel d'aquests casos, estudis entomològics es van dur a terme per conèixer quina espècie de *Culicoides* podia estar actuant com a vector. Els resultats van mostrar la presència de l'espècie *C. imicola* a les illes de Mallorca i Menorca, ampliant així el seu rang de distribució. Abans l'espècie no havia estat detectada a la meitat est d'Espanya, tenint com a límit de distribució més septentrional la localitat de Talavera de la Reina, a Toledo, trobant-se també en altres regions del sud-oest peninsular (Ortega *et al*., 1998; Rawlings *et al*., 1997).

Va ser l'any 2004 quan el virus (serotip 4) es va assentar de manera estable a la meitat sud del país. El primer focus d'aquest serotip es va detectar al sud de la província de Cadis, a pocs quilòmetres del Marroc, on el VLB-4 havia circulat durant els mesos anteriors (Hermoso, 2005; EFSA, 2007). El juliol del 2007 es van detectar els primers casos associats al VLB-1, concretament a Tarifa des d'on es va estendre ràpidament (Allepuz *et al*., 2010). Al novembre del 2007 es detecten focus del serotip 1 al País Basc que s'estenen cap a Galicia i França. El gener del 2008 es detecten els primers casos de serotip 8 a Astúries i Galicia que s'estén a Cantabria (Schwartz-Cornil *et al.*, 2008). A l'octubre del mateix any es van confirmar els primers brots a Andalusia (RASVE, 2013). Les hipòtesis per les possibles vies d'entrada del serotip 8 a la Península inclouen el transport d'animals, semen i embrions o vectors infectats procedents del nord d'Europa.

Amb motiu de l'aparició dels brots de LB els anys 2000 i 2003 a les Illes Balears i de l'any 2004 a Andalusia el *Ministerio de Medio Ambiente y Medio Rural Marino (MARM)* va decidir posar en marxa un Programa Nacional de Vigilància Entomològica específic dins del *Programa Nacional de Erradicación de la Lengua Azul*. L'objectiu del Programa era conèixer les espècies de *Culicoides* associades a explotacions ramaderes amb remugants domèstics. L'atenció es centrava en la detecció de les espècies que es coneixia que estaven implicades en la transmissió de la LB, determinar la seva àrea de distribució, la seva potencial evolució i detectar el seu període d'activitat al llarg de l'any per delimitar els períodes de risc de transmissió de la malaltia (Lucientes *et al*., 2008). Per dur a terme aquesta monitorització es va dividir Espanya en quadrícules de 50Km$^2$, un total de 212 cubrien l'Espanya peninsular, i 13 quadrícules addicionals

per cobrir les Illes Balears, Illes Canaries, Ceuta i Melilla. Inicialment es van col.locar trampes en una quadrícula de cada dos, aquestes trampes funcionaven una nit a la setmana durant tot l'any, constituint el que es coneix com Estacions de Vigilància Entomològica Permanent de la LB. Al llarg dels anys es va anar modificant el plantejament inicial i davant de la necessitat d'ampliar el coneixement de les espècies vectores es va augmentar el nombre de quadrícles on mostrejar, algunes també de forma continuada durant tot l'any i altres com a complement en determinats moments de l'any, denominats mostrejos de reforç.

Els resultats obtinguts durant aquest anys han mostrat que tant l'espècie exòtica *C. imicola* com el complex d'espècies autòctones del grup Obsoletus estan presents a Espanya amb diferents patrons geogràfics. Al nord hi ha major abundància de les espècies del grup Obsoletus i al sud de *C. imicola*. L'espècie *C. pulicaris* es troba repartida per tot el país però amb abundàncies menors que les anteriors.

Aquest Programa ha permès profunditzar en el coneixement de les espècies de *Culicoides* presents al territori espanyol al llarg d'aquests anys (Sarto i Monteys *et al*., 2003; Goldarazena *et al*., 2008; Talavera *et al*., 2011; Alarcón-Elbal i Lucientes, 2012). Aquest fet, juntament amb la crisi econòmica viscuda ha fet que el nombre de quadrícules mostrejades hagi anat disminuint de manera considerable.

Gràcies als mostrejos dels diferents Plans de Vigilància Entomològica de la LB que s'estant portant a terme a molts països europeus, s'ha pogut comprovar que durant els mesos més freds no hi ha activitat dels vectors. Per tant s'ha pogut establir un període de baixa activitat vectorial, més llarg com més fred sigui el clima del territori, definit amb el nom de *vector free period* o període lliure de vectors (referit a *Culicoides*). Poder determinar amb precisió aquest període és molt útil per poder realitzar els moviments del bestiar entre territoris amb garanties de seguretat. L'estratègia utilitzada pel virus de la LB durant aquests mesos de baixes temperatures a dia d'avui no està del tot clara. S'ha vist que després d'aquest temps fred el virus torna a reaparèixer a les mateixes zones on ja havia estat detectat (Osmani *et al.*, 2006). Hi ha diferents teories que intenten explicar com sobreviu el virus aquest temps menys favorable, una de les teories més acceptades és l'establiment d'un període prolongat de virèmia en els remugants (Luedke *et al*., 1977; Takamatsu *et al*., 2003; Napp *et al*., 2011), tot i que en condicions experimentals la màxima duració de la virèmia s'ha observat en el bestiar boví, sent en tots els casos menor dels 100 dies (Sellers i Taylor, 1980). Altres autors plantegen la transmissió

transplacentària en els remugants com una altra via de manteniment del virus durant l'època hivernal (Gibs *et al.*, 1979; Santman-Berends *et al.*, 2010; Takamatsu *et al.*, 2004), proposaven un model basat en l'actuació combinada del vector i l'hoste vertebrat. Es centren en la possibilitat que els limfòcits T γδ que *in vitro* romanen persistentment infectats amb el VLB, es vegin afectats per les proteases derivades de la inflamació de la picada de l'artròpode. Aquests enzims destruirien la càpsida externa del virus de la LB alliberant partícules subvirals considerablement molt més infeccioses (Mertens *et al.*, 1987; Mertens *et al.*, 1996; Hemati *et al.*, 2009), fet que provocaria que aquestes partícules podrien ser activades inclús mesos després de l'última detecció de la virèmia.

## El virus Schmallenberg (VSB)

La malaltia de SB, és una malaltia vírica infecciosa no contagiosa causada per un virus ARN de la familia *Bunyaviridae*, gènere *Orthobunyavirus* i serogrup Simbu, transmesa per vectors, que pot afectar tant a remugants domèstics com salvatges (Hoffman *et al.*, 2012; De Regge *et al.*, 2012; Elbers *et al.*, 2013).

És una malaltia de descobriment molt recent, ja que va ser descrita per primer cop a Alemanya el novembre de l'any 2011. El seu nom "provisional" es deu al nom del poble on es van trobar els primers animals afectats amb la malaltia. Durant l'estiu-tardor del 2011, ramaders i veterinaris de la regió alemanya Renania-Westfalia, en particular d'algunes granges de la localitat de Schmallenberg, van detectar que alguna cosa no anava bé en les seves vaques de llet, tenien febre, diarrea i produïen menys llet. Després de fer totes les proves diagnòstiques disponibles van descartar malalties conegudes que podien estar afectant el bestiar com pestivirosis, llengua blava, febre aftosa, febre de la Vall del Rift entre altres. A continuació es van utilitzar tècniques de metagenòmica per detectar el patogen responsable de la simptomatologia. Es va poder obtenir i ensamblar seqüències d'ARN viral i completar el genoma d'ARN del nou virus Schmallenberg. Els resultats van mostrar que estavem davant d'un nou virus molt semblant al virus Shamonda (Hoffman *et al.*, 2012), un altre *Orthobunyavirus* del serogrup Simbu, aïllat al Japó que afecta també el bestiar boví (Yanase *et al.*, 2012).

La malaltia SB afecta tant a remugants domèstics com salvatges però la incidència de la malaltia en les poblacions afectades és molt baixa. Només un petit percentatge dels animals

infectats presenta algun signe clínic que també varia segons les espècies, com diarrea, febre i baixa producció de llet (Hoffman *et al.*, 2012). Tot i això, uns pocs animals, sobretot ovins, pateixen transtorns greus de la reproducció, produint abortaments i malformacions en el fetus per infecció perinatal (De Regge *et al.*, 2013). Els remugants que superen la infecció queden immunitzats, fet que els protegeix de noves infeccions, raó per la qual la incidència de la malaltia en un determinat territori sol anar disminuint amb el temps (Rodríguez-Prieto *et al*., 2014). Això fa que l'aplicació de vacunes enfront aquesta malaltia no tingui gran interés, excepte per protegir les ovelles reproductores de gran valor.

A l'inici de l'epizoòtia, davant la incertesa de la situació d'emergència d'un nou agent infecciós desconegut, la OIE i la comissió Europea exigien als països afectats declarar tots els brots que es detectaven. Poc després es va comprovar que el virus Schmallenberg no era greu per la sanitat animal ni representava cap risc per la salut pública. Per tant les mesures de control es ven fer més laxes i actualment només és obligatori declarar la primera detecció del virus en el país com a malaltia emergent.

Inicialment es va assumir que els vectors de la malaltia eren dípters del gènere *Culicoides* ja que en altres zones del món són els responsables de la transmissió d'altres virus propers del gènere *Orthobonyavirus* (Jennings i Mellor, 1989; Kurogi *et al*., 1987; Shin *et al*., 2009; Yanase *et al*., 2005). Aquest fet es va avalar en el treball de Rasmussen *et al*. del 2012 on van trobar *Culicoides* capturats l'any 2011 a Dinamarca amb fragments del VSB . De les 130 espècies de *Culicoides* distribuïdes per Europa (Szadziewski i Borkent, 2004 actualitzat 2013; Ramilo *et al*., 2013), fins el moment s'han involucrat 8 espècies de *Culicoides* en la transmissió del VSB, *C. obsoletus*, *C. scoticus*, *C. dewulfi*, *C. chiopterus* (De Regge *et al*., 2012; Elbers *et al*., 2013; Goffredo *et al*., 2013), *C. punctatus (*Larska *et al.,* 2013), *C. pulicaris, C. nubeculosus* i *C. imicola* (Balenghien *et al.*, 2014), coincidents en tots els casos excepte *C. puncatus* i *C. nubeculosus* amb vectors ja coneguts de la LB. Tot i que recentment, s'han trobat indicis que apunten a que les espècies *C. punctatus* i *C. newsteadi* també podrien estar implicades en la transmissió de la LB (Goffredo *et al*., 2015).

Generalment es considera que la distribució i el manteniment de la malaltia de SB, així com totes les arbovirosis, estan associats a unes condicions climàtiques apropiades, la presència d'hostes susceptibles i vectors competents en una mateix moment i lloc (Erasmus i Potgieter, 2009). Després del seu descobriment, ràpidament es va desenvolupar un protocol d'una prova

específica per la detecció ràpida del VSB en animals sospitosos i es va distribuir entre els laboratoris de diagnòstic de sanitat animal de la Unió Europea (Fischer *et al*., 2013). Aquest fet va provocar que en molt poc temps es disparessin els casos detectats de la malaltia per països de tota Europa, com Holanda, Bèlgica, França i Regne Unit, tots ells països veïns d'Alemanya. Tot i això, no es creu que aquestes deteccions fossin degudes a una expansió de la malaltia desde Alemanya sinó que es creu que la malaltia ja feia temps que estava present a la zona però havia passat desapercebuda. Posteriorment va ser detectada a la gran majoria dels països d'Europa arribant als països nòrdics com Finlàndia i Noruega, a Russia per l'est i a Espanya pel sud (Claine *et al.*, 2015).

La primera detecció de SB a Espanya va ser el març del 2012 en una explotació d'ovelles i cabres a la província de Córdoba al sud del país (Rodríguez-Prieto i Sánchez-Vizcaino, 2012). Posteriorment el VSB es va detectar en altres zones del país (Balseiro *et al*., 2013; Astorga *et al*., 2014; Fernández-Aguilar *et al*., 2014) (figura 12).
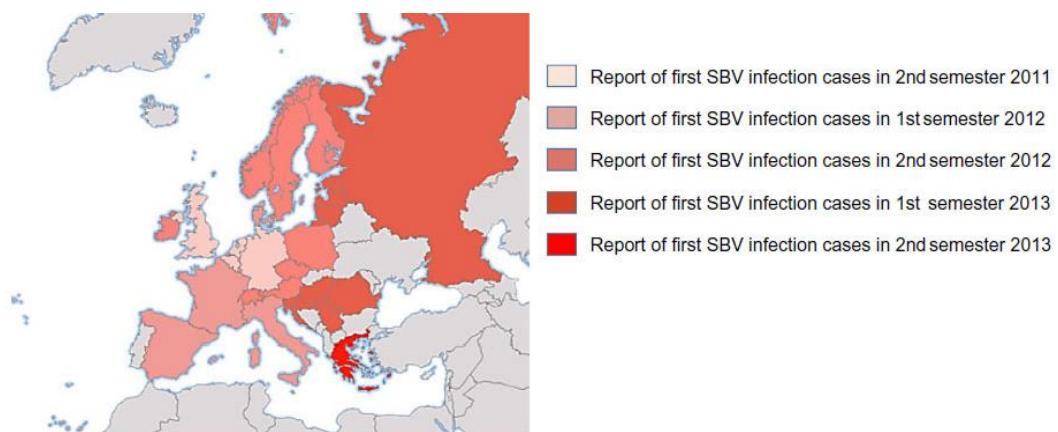


**Figura 12**. Detecció cronològica del VSB a Europa (Adaptat de Claine *et al*., 2015).

**Importància del canvi climàtic en la epidemiologia de la LB i SB**

Existeixen diferents factors que influencien en la prevalença i la distribució de les diferents arbovirosis, entre els que destacariem les condicions ambientals. Els vectors artròpodes són extraordinariament depenents d'unes condicions ambientals específiques que incouen un rang de temperatures, humitat, disponiblitat d'aigua entre altres. Cada espècie de vector ocupa un nínxol ecològic determinat amb unes condicions ambientals concretes, motiu pel qual la seva distribució es pot veure molt afectada per alteracions en aquestes. Per tant les arbovirosis en general són considerades com malalties extremadament sensibles al canvi climàtic (Jiménez Clavero, 2009).

L'escalfament global (+0.6ºC en l'últim mig segle), és una realitat que fins els més escèptics reconeixen objectivament. Aquest fenòmen va en augment, de manera que durant el segle XXI s'espera un increment mig de la temperatura d'entre +1.6 i +6ºC. També sembla que existeix consens entre la comunitat científica sobre el motiu d'aquest augment de temperatures, la principal causa sembla ser antropogènica, especialment per les emissions de gasos d'efecte hivernacle, activitat industrial, consum de combustibles fòssils i producció d'energia entre altres. Es preveu que aquest augment de les temperatures vagi acompanyat de més sequeres, més incendis forestals i onades de calor extremes (Jiménez Clavero, 2009) (Prediction of Regional scenarios and Uncertainties for Defining EuropeaN Climate change risks and Effects).

La bilogia dels *Culcioides* està directament relacionada amb la temperatura ambiental, ja que el seu metabolisme és incapaç de proporcionar-los una temperatura constant que els permeti estar actius de forma continuada. Els *Culicoides* estan actius només en un rang determinat de temperatures característic de cada espècie (Wittmann i Baylis, 2000). Les temperatures òptimes depenen de cada espècie però es troben en un rang de 25 a 30ºC. Tot i així temperatures compreses entre els 18 i 30ºC els són adequades per poder realitzar el seu cicle biològic, i el cicle de transmissió dels diferents patògens.Temperatures més baixes inhibeixen la seva activitat, entre 0 i 10ºC deixen de volar i alimentar-se però no moren sinó que romanen en un estat de diapusa hivernal, començarien a morir si les temperatures es mantinguessin durant cert temps per sota de 0ºC (Lucientes, 2014).

El canvi climàtic que estem vivint, provocarà hiverns més suaus, que no només reduiran la mortalitat de les larves, sinó que adelantaran el seu desenvolupament, per tant els adults començaran abans el seu període d'activitat, allargant-lo. Pot produir a més un increment de la competència vectorial en els *Culicoides* i inclús induir l'anomenada competència en espècies no competents. En condicions de camp només una fracció dels individus de la població són capaços de transmetre la malaltia, en la resta d'individus existeix un o més sistemes de barrera que sembla impedir l'inici de la infecció o transmissió del virus mes enllà de les cèl.lules intestinals (Mellor, 1990; Mellor *et al*., 2000). Diferents estudis han comprovat que un augment de 5-10ºC en la temperatura durant el desenvolupament larvari, provoca l'augment de la taxa d'infecció dels adults (Mellor *et al*., 1998).

En el cas concret de la Península Ibèrica, es creu que l'augment de les temperatures afavorirà que *C. imicola*, colonitzi noves zones, podent arribar fins i tot a altres països d'Europa i introduir-se en nínxols ecològics fins ara ocupats per altres vectors o substituir altres espècies més adaptades al fred (Lucientes, 2014). En contrapartida, altres espècies vectores més adaptades als climes més freds i humits com les espècies del grup Obsoletus i *C. pulicaris* podrien restringir la seva àrea de distribució a zones situades més al nord o zones de major alçada. Però no només pot variar la distribució de les espècies vectores ja conegudes sinó que altres espècies que fins ara no es contemplaven en els estudis de risc epidemiològic, podrien modificar la seva capacitat vectorial i convertir-se en nous vectors (Lucientes, 2014). S'ha vist que algunes espècies de *Culicoides* no vectores, al criar-se en laboratori a temperatures uns graus més elevades són capaces d'infectar-se (Mellor, 2000; Mellor *et al*., 2000)

A part de l'augment de les temperatures el canvi climàtic pot comportar altres canvis com una disminució de la pluviositat anual que pot provocar una alteració en els períodes de pluja amb fenòmens extrems de precipitacions i grans períodes de sequera, condicions que poden afectar el desenvolupament dels estadis immdurs i l'activitat dels adults podent causar un augment en la mort dels vectors (Lucientes, 2014).

Per tant, la variabilitat en la probabilitat de transmissió de les diferents malalties per part dels *Culicoides* degut al canvi climàtic*,* dependrà de la interacció entre els efectes antagonistes de tots aquests canvis que poden experimentar les condicions ambientals (Wilson i Mellor, 2009).

**Objectius**

## Objectius

Amb la intenció de profunditzar en el coneixement de les espècies del gènere *Culicoides* i de les relacions hoste-vector que s'estableixen en les malalties de la LB i SB, els principals objectius d'aquesta tesi doctoral han estat quatre:

1. Definir la diversitat i distribució d'espècies del gènere *Culicoides* a Catalunya, especialment d'aquelles espècies proposades com a potencials vectors de la LB i SB.
2. Caracteritzar la presència d'espècies críptiques dins el subgènere *Culicoides.*
3. Caracteritzar les comunitats de *Culcoides* presents en ecosistemes naturals amb presència de remugants salvatges.
4. Determinar les preferències d'hoste de les comunitats de *Culicoides* presents en ecosistemes naturals amb presència de remugants salvatges.

Per aconseguir aquests objectius, s'han realitzat una sèrie de treballs que engloben la present tesi. L'estructuració és la següent:

El Capítol I estudia la diversitat i distribució de les espècies de *Culicoides* a Catalunya, amb aquest objectiu s'han integrat dues metodologies, la morfologia clàssica i la biologia molecular, per aconseguir una identificació més precisa de les diferents espècies. També s'ha realitzat un estudi de la seva distribució a l'àrea d'estudi analitzant la possible influència de diferents variables bioclimàtiques.

**Sandra Talavera** , Francesc Muñoz-Muñoz & Nonito Pagès (2011) New insights on diversity, morphology and distribution of *Culicoides* Latreille 1809 (Diptera:Ceratopogonidae) from Northeast Spain, Annales de la Société entomologique de France (N.S.), 47:1-2, 214-231, DOI: 10.1080/00379271.2011.10697714

El Capítol II es centra en l'anàlisi exhaustiu d'individus pertanyents a les espècies *C.pulicaris* i *C. punctatus* capturats en diferents àrees d'Espanya. Utilitzant la metodologia combinada esmentada al capítol anterior, es vol saber si dins aquestes espècies existeixen espècies críptiques, fet rellevant degut a que són espècies de reconeguda importància veterinària perquè poden actuar com a transmissors de diferents malalties.

**Talavera S**, Muñoz-Muñoz F, Verdún M, Pagès N (1st review) Morphology and DNA barcode reveals three species in one: Description of *Culicoides cryptipulicaris* sp. nov and *Culicoides quasipulicaris* sp. nov in the subgenus *Culicoides* (Diptera: Ceratopogonidae). Parasites and Vectors

El tercer estudi (Capítol III) es centra en el coneixement de les comunitats de *Culicoides* presents en els ecosistemes naturals amb remugants salvatges a Espanya. Centrant el nostre interés en les espècies de *Culicoides* conegudes com a vectors de la LB i SB, realitzant una comparativa de la distribució d'aquestes espècies en zones naturals de remugants salvatges i en zones properes de granges de remugants domèstics, per veure si concideix i poder saber si aquestes espècies poden estar actuant com a espècies pont de diferents patògens entre els remugants domèstics i salvatges.

**Talavera S**, Muñoz-Muñoz F, Durán M, Verdún M, Soler-Membrives A, Oleaga Á, et al. (2015) *Culicoides* Species Communities Associated with Wild Ruminant Ecosystems in Spain: Tracking the Way to Determine Potential Bridge Vectors for Arboviruses. PLoS ONE 10(10): e0141667. doi:10.1371/journal.pone.0141667

Una vegada conegudes les comunitats de *Culicoides* presents en zones naturals amb remugants salvatges el nostre objectiu en el Capítol IV ha estat confirmar que aquestes espècies s'alimenten de remugants salvatges. Mitjançant eines moleculars, s'ha identificat la procedència de la sang present en els abdomens dels *Culicoides* per saber de quins hostes s'han alimentat i així validar la hipòtesi llançada en el Capítol anterior.

**Talavera S** , Verdún M, Pujol N, Muñoz-Muñoz F, Pagès N (2015) (In process) *Culicoides* host-feeding preferences in natural ecosystems in Spain. PLoS ONE

**Capítol I**

# New insights on diversity, morphology and distribution of *Culicoides* Latreille 1809 (Diptera: Ceratopogonidae) from Northeast Spain

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**Abstract.** The present work provides an improvement on the knowledge of the diversity, morphology and distribution of *Culicoides* species in Catalonia (NE Spain). Data derived from morphology and sequencing allowed to detect the presence of 13 species non previously found in the surveillance area, updating the number of species recorded in Catalonia up to 53. Of special interest among the newly recorded species are: 1) *C. yemenensis*, new record for Europe, 2) *C. coluzzii* and *C. sejfadinei*, being new records for the Iberian Peninsula, and 3) *C. pseudopallidus* which is new record for Spain. Regarding the current distribution of *Culicoides* species, two eco-zones were detected in the region, the first eco-zone had species with typical northern Palaearctic (European) distribution whereas the second had species with typical southern Palaearctic (African) distribution, which were apparently influenced by mean high temperature of the warmest month and annual precipitation rate.

**Résumé. Nouvel aperçu de la diversité, de la morphologie et de la distribution des *Culicoides* Latreille 1809 (Diptera : Ceratopogonidae) du nors-est de l'Espagne.** Le présent travail présente une amélioration de la connaissance de la diversité, de la morphologie et de la distribution des espèces de *Culicoides* en Catalogne (NE Espagne). Les données obtenues grâce à la morphologie et au séquençage ont permi de détecter la présence de 13 espèces auparavant non connues dans l'aire d'étude, élevant ainsi le nombre d'espèces de la Catalogne à 53. Parmi ces nouvelles données, les suivantes ont un intérêt particulier : 1) *C. yemenensis*, première observation en Europe ; 2) *C. coluzzii* et *C. sejfadinei*, premières observations dans la péninsule Ibérique ; 3) *C. pseudopallidus*, première observation en Espagne. En ce qui concerne la distribution actuelle des espèces de *Culicoides*, deux éco-zones ont été détectées dans la région, la première de ces éco-zones a des espèces typiquement nord-paléarctiques (Européennes) tandis que la deuxième a des espèces typiquement sud-paléarctiques (Africaines), ces dernières étant influencées par la moyenne des températures maximales et par les précipitation annuelles totales.

**Keywords:** Catalonia, bluetongue, distribution, morphology, diversity.

Biting midges of the genus *Culicoides* Latreille (1809) are distributed almost worldwide excluding the Antarctica and New Zealand. All around the world more than 1340 species of *Culicoides* (Diptera: Ceratopogonidae) have been described and classified among 39 subgenera (Borkent & Wirth 1997; updated 2008). *Culicoides* midges are amongst the smallest haematophagous dipteran insects being 1–3 mm in size, and are well known as disease transmitters, being vectors for protozoa, filarial worms and overall many viruses affecting humans and domestic or wild animals (Mellor *et al.* 2000).

Bluetongue (BT) is one of the most economically important diseases transmitted by *Culicoides* producing high economic losses and disruption in both international and domestic trade (Purse *et al.* 2005). The economical impact of BT-8 outbreaks in Northwest-

ern Europe was estimated over 150M € (Hoogendam 2007). BT is an infectious non-contagious disease of domestic and wild ruminants being transmitted exclusively by the bites of infected competent midges (Du Toit 1944; Mellor & Pitzolis 1979). The disease is restricted into areas where competent species do occur. In the last decade BT re-emerged in the Mediterranean basin and since then several serotypes have been circulating across many Mediterranean countries. Initially, the spread of the disease was associated with the introduction and establishment of the exotic species *Culicoides imicola* Kieffer (1913) into European Mediterranean countries. Once the disease was established, few endemic species of *Culicoides* were described as potential vectors for the disease according to viral isolations (Caracappa *et al.* 2003; De Liberato *et al.* 2005; Savini *et al.* 2005). After that the unexpected appearance of BT serotype 8 in Northwestern Europe, where *C. imicola* was not present, confirmed the involvement of endemic Palaearctic species in BT transmission. Species in the Avaritia subgenus as *C. dewulfi* Goetghebuer (1936) and *C. chiopterus* (Meigen 1830) were then de-

scribed as new BT vectors based on virus detection by RT-PCR (Meiswinkel *et al.* 2007; Dijkstra *et al.* 2008), whereas *C. obsoletus* and *C. scoticus* were confirmed as BT vectors in experimental infection studies (Carpenter *et al.* 2008).

As a result of the current BT epidemics and the suspected involvement of endemic *Culicoides* species in viral transmission many European countries concentrate efforts towards surveillance, being the entomological monitoring one of the key issues. Entomological surveillance focus on two main objectives: i) to improve the knowledge on diversity of *Culicoides* species, which depends on accurate identification, and ii) to improve the knowledge of *Culicoides* species distribution, specially those which are suspected to be potential vectors of the disease.

In the present work are depicted results on *Culicoides* species identification and distribution as a result of the current Entomological Surveillance Program performed in the Autonomous Community of Catalonia which is located in the northeast part of Spain, a region recently affected by several bluetongue serotype 1 outbreaks.

## Material and methods

### Sampling

*Culicoides* specimens identified in the study were trapped between years 2003–2008 throughout the Bluetongue Entomological Surveillance Program developed in Catalonia (NE Spain). *Culicoides* were trapped by means of CDC black light traps (John W. Hock Company) placed in farms settled with either sheep or goats, being this trap model the one provided for
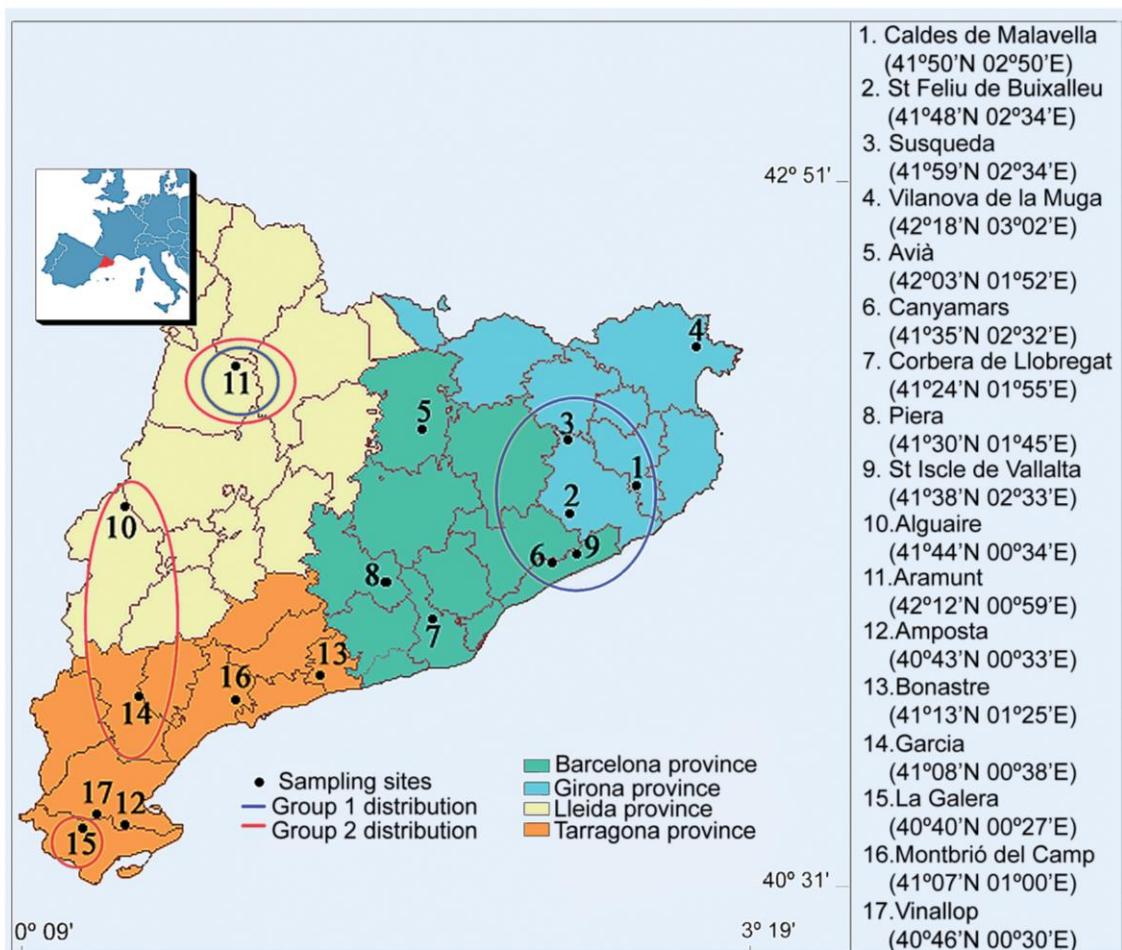


**Figure 1**
Map of Catalonia showing sampling sites distribution among the four provinces and distribution of *Culicoides* species belonging to Group 1 and 2.

the administration to perform the Entomological Surveillance Program. One CDC trap was placed outside the animal's enclosure at each of the 17 farms sampled and was operated once a week throughout the year, being switched on at dusk and off at dawn the following day. The farms were distributed among the surveillance area, from coastline locations to mountainous areas, being coastline and inner close locations of special interest. The region is divided into four geographical units or provinces: i) Girona province (G) with four sampling sites numbered: Caldes de Malavella (1), Sant Feliu de Buixalleu (2), Susqueda (3), Vilanova de la Muga (4); ii) Barcelona province (B) with five sites numbered: Avià (5), Canyamars (6), Corbera de Llobregat (7), Piera (8), Sant Iscle de Vallalta (9); iii) Lleida province (L) with two farms numbered: Alguaire (10), Aramunt (11); and iv) Tarragona province (T) with six sites numbered: Amposta (12), Bonastre (13), Garcia (14), La Galera (15), Montbrió del Camp (16) and Vinallop (17); (fig. 1).
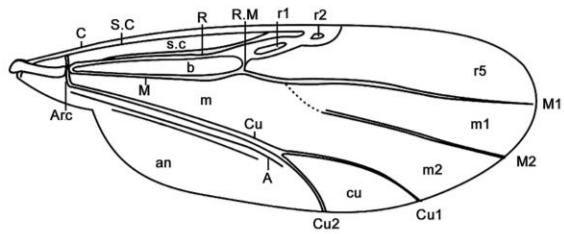


**Figure 2**
Scheme of the *Culicoides* wing parts, differentiating i) veins: C, costa; SC, subcostal; R, radial; M, M1, M2, median and branches; Cu, Cu1, Cu2, cubital and branches; A, anal; RM, radial-median crossvein; Arc, arculus; and ii) cells: b, basal; sc, subcostal; r1, first radial; r2, second radial; r5, fifth radial; m, m1, m2, medians; cu, cubital; an, anal.
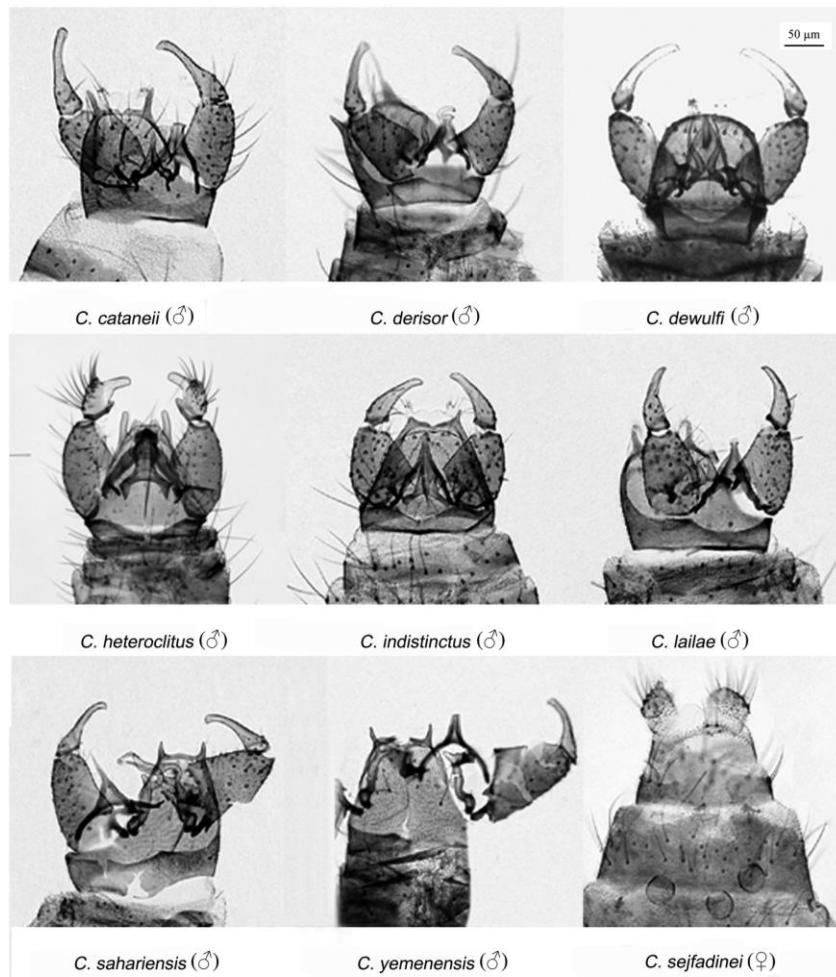


**Figure 3**
Pictures of *Culicoides* genitalia and spermathecae.

**Morphological identification**

Trapped insects were killed by freezing them a minimum of 60 minutes at -20 °C, and stored in ethanol for further identification. *Culicoides* midges were first identified under a stereomicroscope (Nikon SMZ) by morphology at species-group level according to wing pattern, characterized by markings distributed among different regions of the wing (fig. 2). Then individuals were dissected and body midge was fixed on microscope slides but a small part of the body midge, usually two legs and the anterior part of the abdomen was stored in ethanol 96% at -20 °C and processed for the molecular identification based on the sequentiation of a region of 472bp from the mitochondrial gene Cytochrome Oxidase subunit I (COI).

An accurate morphological identification at species-level was performed in all individuals cited in the present work by examination of slide mounted specimens using a Nikon Eclipse E200 light microscope. Several metric traits were measured and recorded in all species to avoid data misinterpretation (Appendices 1–2), as some disparity has been observed in the methodology used by different authors. Non metric traits were also recorded when available, as was the male genitalia which has been shown a valuable diagnostic character to distinguish species (fig. 3). The wings of all species identified in the study were photographed and colour-contrasted *in silico* to analyze the common wing pattern of each species in detail (fig. 4).

The most relevant bibliographic resources dealing with general identification of *Culicoides* were used to morphologically diagnose all midges. The taxonomic keys of Campbell & Pelham-Clinton (1960), Kremer (1965), Delécolle (1985) and Glukhova (1989) were used, as well as other published specific papers further listed in the results and discussion section. Notes on the known distribution of the listed species were inferred from published literature as well as from Fauna Europea database (www.faunaeur.org).

**Distribution Analyses**

The distribution of two groups of *Culicoides* species in the surveillance area was studied to analyze the possible influence of bioclimatic variables. Group 1 with species characterized by an European distribution with non detected records from Africa, trapped exclusively in some of the eastern locations and Aramunt and Group 2 with typically African species that in Europe are only present in warmer Mediterranean regions, trapped exclusively in some of the western locations (fig. 1). Four variables

were included in the analysis: annual precipitation rate, mean low temperature of the coldest month, mean high temperature of the warmest month, and altitude above sea level (a.s.l.) of sampling sites. All data was obtained from the database *Atles climàtic digital de Catalunya* (http://magno.uab.es/atles-climatic/) (fig. 5), unless altitude which was obtained through GPS.

We used *Miramón* software (Pons 2000) as geographic information system (GIS) tool to obtain variables values in each of the 9 sampling sites where either *Culicoides* belonging to Group 1 or Group 2 were present (tab. 1). The *Hierarchical cluster analysis* (SPSS Inc 2006) was use to generate different farm dendrograms according to similarities for each of the bioclimatic variables considered (fig. 6). Data belonging to Aramunt, the only location where both groups of *Culicoides* were present, was excluded from the analysis to ascertain those bioclimatic variables having differences between the groups.

## Results and discussion

In the present study, among a total of 258840 trapped specimens, 45 different species of the genus *Culicoides* have been morphologically identified in the surveillance area, some of them being close to other species belonging to species groups. In all cases species identification was supported by the molecular identification of all examined individuals through sequencing.

Concerning *Culicoides* diversity, we reported up to 13 new recorded species since the last *Culicoides* faunistic study performed in the region (Sarto i Monteys *et al.* 2009). All of them represent new records for the surveillance area, whereas few of them were new records at different geographical scale: i) *C. yemenensis* Boorman (1989) was a new record for Europe, ii) *C. coluzzii* Callot *et al.* (1970) and *C. sejfadinei* Dzhafarov (1958) were new records for the Iberian Peninsula, iii) *C. pseudopallidus* Khalaf (1961) was new record for Spain and iv) 9 species were new records for Catalonia, being previously recorded in Spain (tab. 2).

### Species remarks

The following listed species are those representing new records, as those recently reported (Sarto i Monteys

**Table 1.** Data summary of bioclimatic variables analyzed and presence of *Culicoides* species from Group 1 and 2 at each sampling site. A, altitude; AP, annual precipitation; LT, mean low temperature of the coldest month; HT, mean high temperature of the warmest month.

| Presence/absence | Sampling Site | Coordinates | A (m) | AP (mm) | LT (°C) | HT (°C) |
|---|---|---|---|---|---|---|
| Group1 / Group2 | Caldes de Malavella | 41°50'N 02°50'E | 120 | 793.2 | 2.4 | 29.6 |
| Group1 / Group2 | Sant Feliu de Buixalleu | 41°48'N 02°34'E | 419 | 835.1 | 1 | 27.9 |
| Group1 / Group2 | Susqueda | 41°59'N 02°32'E | 349 | 916.8 | 0.7 | 29.5 |
| Group1 / Group2 | Canyamars | 41°35'N 02°27'E | 240 | 739 | 3 | 28.3 |
| Group1 / Group2 | Sant Iscle de Vallalta | 41°38'N 02°33'E | 227 | 805.5 | 3.5 | 27.5 |
| Group2 / Group1 | Alguaire | 41°44'N 00°34'E | 327 | 445.2 | −0.3 | 32 |
| Group2 / Group1 | La Galera | 40°40'N 00°27'E | 112 | 620.9 | 3.9 | 31.9 |
| Group2 / Group1 | Garcia | 41°08'N 00°38'E | 65 | 427.3 | 2.7 | 32.8 |
| Group1 and Group2 | Aramunt | 42°12'N 00°59'E | 559 | 678.2 | −2.5 | 30.6 |

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**Table 2.** Current list of *Culicoides* species recorded in Catalonia and species distribution at province level.

E, new record for Europe; IP, new record for Iberian Peninsula; S, new record for Spain; C, new record for Catalonia; B, Barcelona; G, Girona; L, Lleida; T, Tarragona.

| | New records | Known distribution |
|---|---|---|
| 1. *Culicoides (Silvaticulicoides) achrayi* Kettle & Lawson 1955 | | B, G |
| 2. *Culicoides alazanicus* Dzhafarov 1961 | | G |
| 3. *Culicoides begueti* Clastrier 1957 | | B, G, L |
| 4. *Culicoides (Oecacta) brunnicans* Edwards 1939 | | G |
| 5. *Culicoides cataneii* Clastrier 1957 | | B, G, T |
| 6. *Culicoides (Beltranmyia) circumscriptus* Kieffer 1918 | | B, G, L, T |
| 7. *Culicoides coluzzii* Callot, Kremer & Bailly –Choumara 1970 | IP | L, T |
| 8. *Culicoides (Culicoides) deltus* Edwards 1939 | | L |
| 9. *Culicoides derisor* Callot & Kremer 1965 | | T |
| 10. *Culicoides (Avaritia) dewulfi* Goetghebuer 1936 | | B, G, L |
| 11. *Culicoides (Culicoides) fagineus* Edwards 1939 | | B, G, L, T |
| 12. *Culicoides (Silvaticulicoides) fascipennis* (Staeger 1839) | | L |
| 13. *Culicoides festivipennis* Kieffer 1914 | | B, G, L |
| 14. *Culicoides (Culicoides) flavipulicaris* Dzhafarov 1964 | | B, G, L, T |
| 15. *Culicoides furcillatus* Callot, Kremer & Paradis 1962 | | B, G, L |
| 16. *Culicoides gejgelensis* Dzhafarov 1964 | C | B, G, L |
| 17. *Culicoides griseidorsum* Kieffer 1918 | | G |
| 18. *Culicoides heteroclitus* Kremer & Callot 1965 | | G, T |
| 19. *Culicoides (Avaritia) imicola* Kieffer 1913 | | B, G, L, T |
| 20. *Culicoides (Culicoides) impunctatus* Goetghebuer 1920 | | G |
| 21. *Culicoides indistinctus* Khalaf1961 | C | T |
| 22. *Culicoides jumineri* Callot & Kremer 1969 | | B, G, L, T |
| 23. *Culicoides kibunensis* Tokunaga 1937 | | B, G |
| 24. *Culicoides kurensis* Dzhafarov 1960 | | G, L, T |
| 25. *Culicoides lailae* Khalaf 1961 | C | L |
| 26. *Culicoides (Oecacta) longipennis* Khalaf 1957 | | G, T |
| 27. *Culicoides lupicaris* Downes & Kettle 1952 | C | G |
| 28. *Culicoides maritimus* Kieffer 1924 | | B, G, T |
| 29. *Culicoides (Wirthomyia) minutissimus* (Zetterstedt 1855) | | G |
| 30. *Culicoides (Culicoides) newsteadi* Austen 1921 | | B, G, L, T |
| 31. *Culicoides (Avaritia) obsoletus* (Meigen 1818) | | B, G, L, T |
| 32. *Culicoides odiatus* Austen 1921 | | L |
| 33. *Culicoides (Silvaticulicoides) pallidicornis* Kieffer 1919 | | B, G |
| 34. *Culicoides (Monoculicoides ) parroti* Kieffer 1922 | | B, G, L |
| 35. *Culicoides pictipennis* (Staeger 1839) | | T |
| 36. *Culicoides (Silvaticulicoides) picturatus* Kremer & Deduit 1961 | C | B |
| 37. *Culicoides poperinghensis* Goetghebuer 1953 | C | B |
| 38. *Culicoides pseudopallidus* Khalaf 1961 | S | L, T |
| 39. *Culicoides (Culicoides) pulicaris* (L. 1758) | | B, G, L, T |
| 40. *Culicoides (Culicoides) punctatus* (Meigen 1804) | | B, G, L, T |
| 41. *Culicoides (Monoculicoides) puncticollis* (Becker 1903) | | G, L |
| 42. *Culicoides (Pontoculicoides) saevus* Kieffer 1922 | | L |
| 43. *Culicoides (Oecacta) sahariensis* Kieffer 1923 | | L, T |
| 44. *Culicoides (Avaritia) scoticus* Downes & Kettle 1952 | | B, G, L, T |
| 45. *Culicoides (Pontoculicoides) sejfadinei* Dzhafarov 1958 | IP | L |
| 46. *Culicoides simulator* Edwards 1939 | | G |
| 47. *Culicoides (Culicoides) subfagineus* Delécolle & Ortega 1998 | | G |
| 48. *Culicoides (Silvaticulicoides) subfasciipennis* Kieffer 1919 | | B, G |
| 49. *Culicoides submaritimus* Dzhafarov 1962 | C | G |
| 50. *Culicoides (Oecacta) truncorum* Edwards 1939 | C | B |
| 51. *Culicoides univittatus* Vimmer 1932 | | G |
| 52. *Culicoides (Oecacta) vexans* (Staeger 1839) | C | G |
| 53. *Culicoides yemenensis* Boorman 1989 | E | L |

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New insights of *Culicoides* from NE Spain

et al. 2009), in the surveillance area. Information provided for each species includes the number of individuals both morphologically and molecularly examined, key notes on morphological diagnosis and notes on their known general wide distribution. Additionally, a list with the recorded distribution of all species in Catalonia is given in table 2, where some of the individuals were only morphologically identified.

### Culicoides (Oecacta) brunnicans Edwards 1939

**Material.** Susqueda (G): 02.VI.2005 1♀.

A single female was trapped throughout the study period, and the morphological observations were in accordance with those descriptions present in Kremer (1965).

**Known distribution:** Britain Is., Channel Is., Czech Republic, Danish mainland, French mainland, Germany, Ireland, Italian mainland, Northern Ireland, Portuguese mainland, Slovakia, Spanish mainland and Near East.



*C. achrayi* (♀)    *C. alazanicus* (♀)    *C. begueti* (♂)

*C. brunnicans* (♀)    *C. cataneii* (♀)    *C. circumscriptus* (♀)

*C. coluzzii* (♀)    *C. derisor* (♂)    *C. dewulfi* (♂)

*C. fagineus* (♀)    *C. festivipennis* (♀)    *C. flavipulicaris* (♀)

*C. furcillatus* (♀)    *C. gejgelensis* (♀)    *C. griseidorsum* (♀)

500 µm
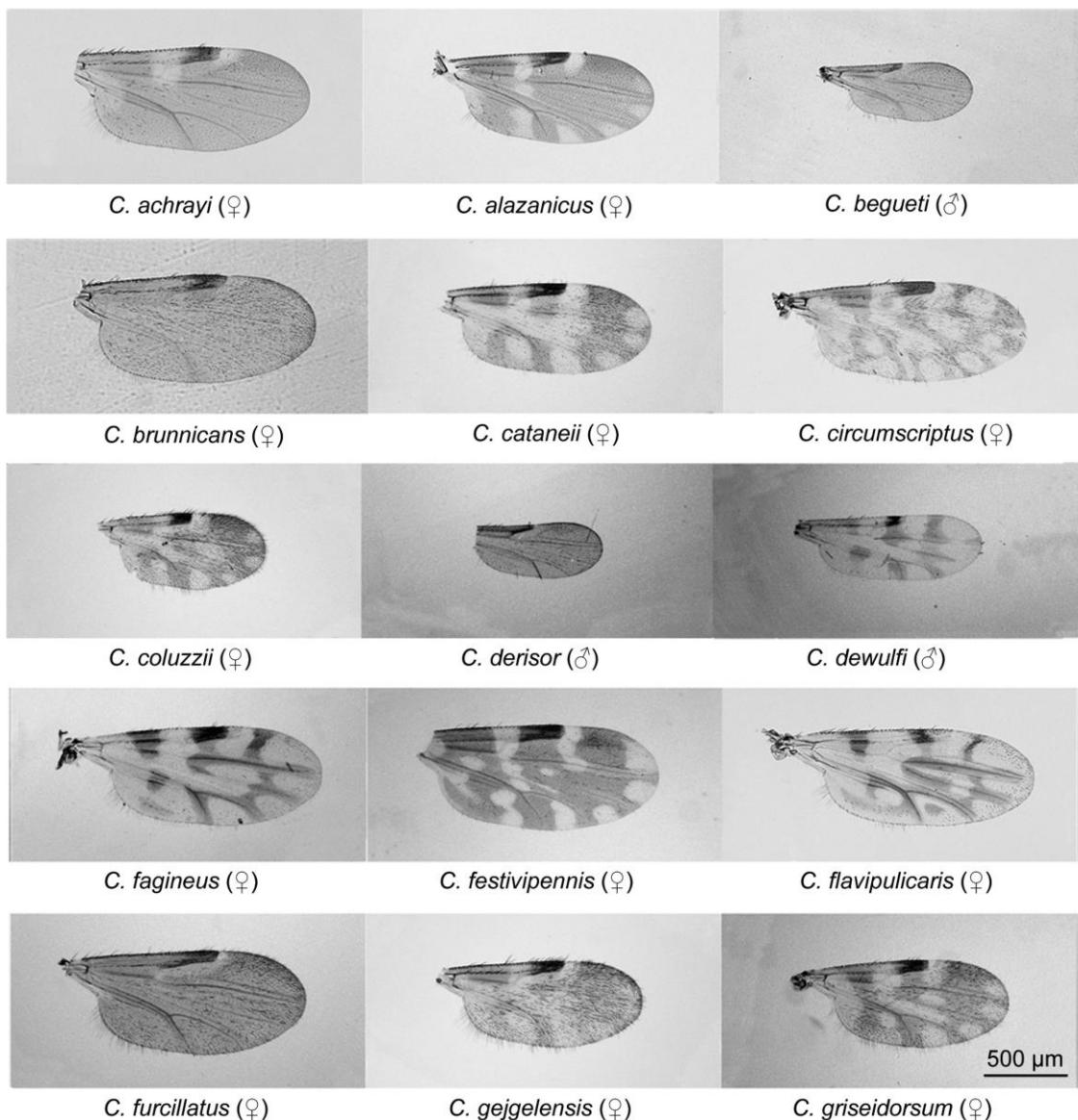
**Figure 4**
Wing pattern pictures of each *Culicoides* species identified.

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## *Culicoides coluzzii* Callot, Kremer & Bailly-Choumara 1970

**Material.** Alguaire (L): 25.VIII.2005 1♀, 01.IX.2005 1♀; La Galera (T): 01.IX.2005 1♀.

Morphological characters of female specimens were in accordance with Callot *et al.* (1970). The wing pattern remained essential to distinguish *C. coluzzii* from other species of the Similis group because: i) *C. coluzzii* has one spot close to r2, whereas *C. similis* has either two spots or a bilobulated spot, and ii)

the m1 spot did contact veins M1 and M2, whereas the spot did not contact the veins in *C. sahariensis* (Callot *et al.* 1970) (fig. 4). Wing pattern differences were enough to detect an misidentified *C. sahariensis* female (La Galera 01.IX.2005; Sarto i Monteys *et al.* 2009) which was confirmed to be *C. coluzzi* by molecular biology results in the present examination.

Based on our results, where morphological and molecular differences were detected in the examined specimens, the synonymy between *C. coluzzii* and *C. sahariensis* proposed by some authors (Boorman *et al.* 1989; Rawlings 1996; Dik *et al.*
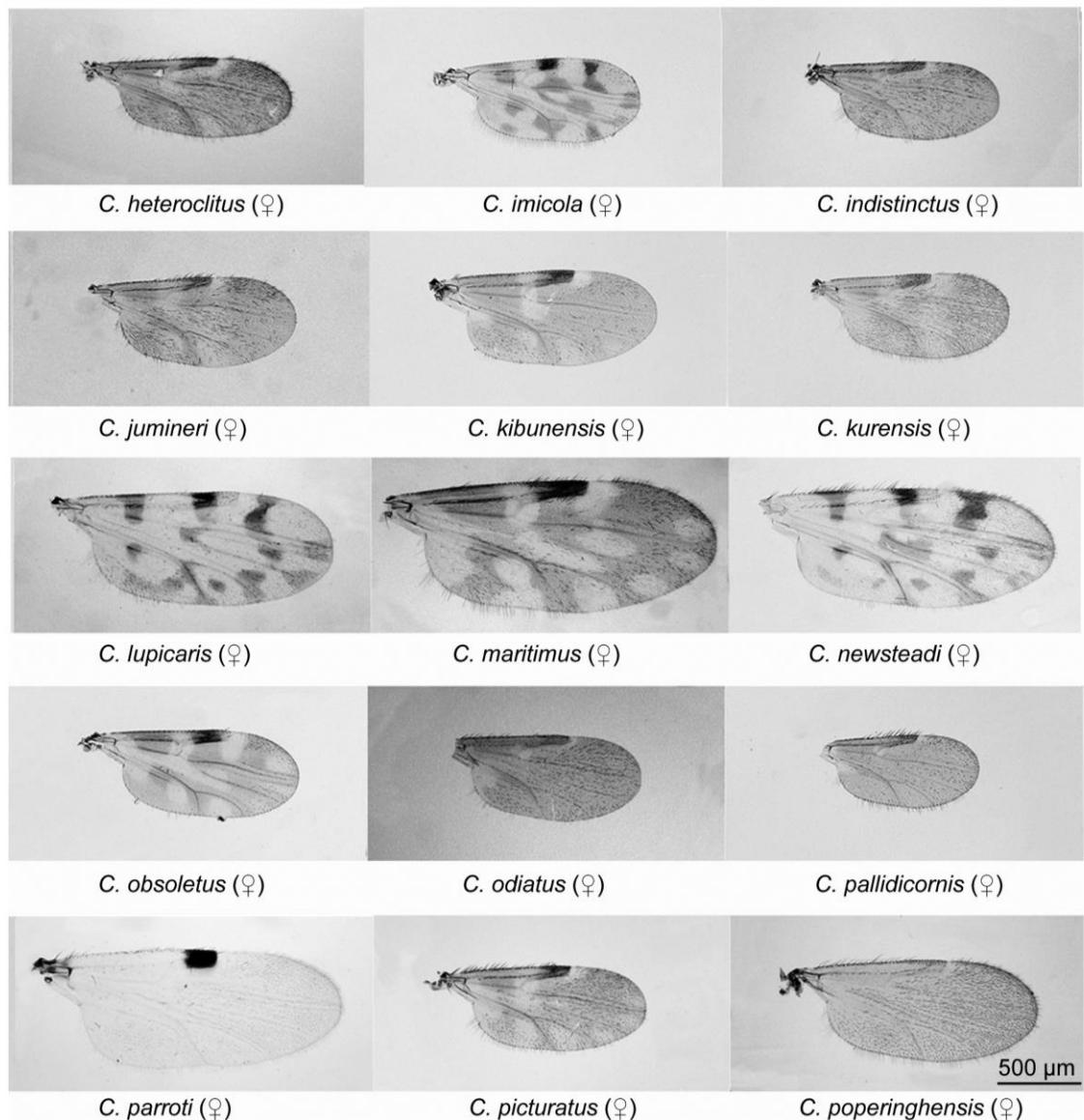


**Figure 4 (Continued)**
Wing pattern pictures of each *Culicoides* species identified.

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2006) can not be supported and we consider this issue should be further revised using type material if possible.

**Known distribution:** Cyprus, Italian mainland and North Africa.

### *Culicoides derisor* Callot & Kremer 1965

**Material.** Garcia (T): 10.VIII.2006 1♂.

A single male specimen was trapped throughout the study

period, and all morphological characters recorded were in accordance with those described in Callot & Kremer (1965).

**Known distribution:** Corsica, French mainland, Portuguese mainland, Spanish mainland, Near East and North Africa.

### *Culicoides (Avaritia) dewulfi* Goetghebuer 1936

**Material.** Sant Feliu de Buixalleu (G): 24.X.2008 1♂; Aramunt (L): 30.X.2008 1♀.
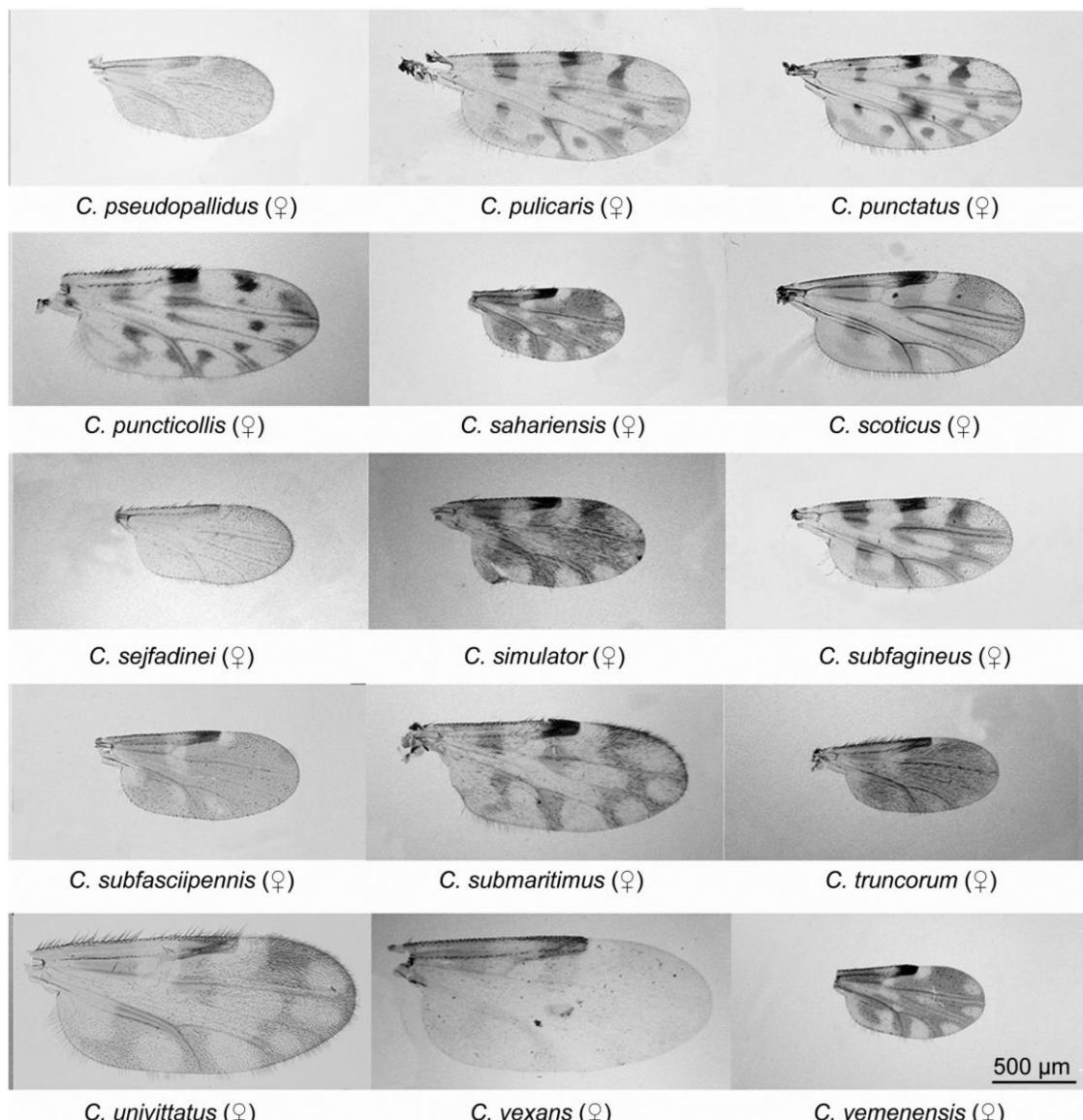


**Figure 4 (Continued)**
Wing pattern pictures of each *Culicoides* species identified.

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Female specimens were similar to other species of the Obsoletus group, main characters differentiating *C. dewulfi* females were the unequal size of the two spermathecae, and a bigger pale spot in the wing cell r5 (Delécolle 1985). Despite these characters are usually used in differentiating the species, a valid threshold value has not been defined because intraspecific variability has not been assessed. Therefore the male specimen analyzed, with highly characteristic genitalia (fig. 3), was useful to confidently identify the species, and further sequencing confirmed the female analyzed were *C. dewulfi*.

**Known distribution:** Belgium, Britain Is., Channel Is., Czech Republic, Estonia, French mainland, Germany, Ireland, Italian mainland, Northern Ireland, Norwegian mainland, Poland, Romania, Russia, Slovakia, Spanish mainland, Turkey, Ukraine, East Palaearctic and Near East.

## *Culicoides (Culicoides) fagineus* s.l. Edwards 1939

**Material.** Susqueda (G): 31.VIII.2006 4♀♀; Garcia (T): 24.V.2007 1♀, 31.V.2007 2♀♀, 21.VI.2007 1♀.

Species closely-related to *C. subfagineus* (Delécolle *et al.* 1998) and both present in the area analyzed. All four specimens analyzed are in agreement with *C. fagineus* description according to Delécolle *et al.* (1998), however further molecular analyses using more individuals revealed the presence of two cryptic sympatric species (Pagès *et al.* 2009).

**Known distribution:** Distribution of the species can be biased due to historical misidentification with *C. subfagineus* Delécolle *et al.* (1998). Britain Is., Corsica, Czech Republic, Danish mainland, Estonia, French mainland, Germany, Italian mainland, Lithuania, Portuguese mainland, Russia, Slovakia, Spanish mainland, Turkey, Ukraine, East Palaearctic, Near East and North Africa.

## *Culicoides festivipennis* Kieffer 1914 (syn. *C. odibilis* Austen 1921)

**Material.** Sant Iscle de Vallalta (B): 23.VI.2008 1♀; Vilanova de la Muga (G): 18.XI.2004 1♀.

The species can be distinguished from *C. clastrieri* by sensillae coeloconia distribution (SD), the latter without sensillae coeloconica from the fourth antennal segment until the tenth (Kremer 1965).

**Known distribution:** Austria, Belarus, Belgium, Bosnia and Herzegovina, Britain Is., Channel Is., Corsica, Croatia, Czech Republic, Danish mainland, Estonia, French mainland, Germany, Hungary, Ireland, Italian mainland, Lithuania, Norwegian mainland, Poland, Portuguese mainland, Russia, Slovakia, Spanish mainland, Switzerland, The Netherlands, Turkey, Ukraine, East Palaearctic, Near East and North Africa.

## *Culicoides furcillatus* Callot Kremer & Paradis 1962

**Material.** Canyamars (B): 28.VII.2005 1♀, Sant Iscle de Vallalta (B): 26.VI.2008 1♀; Susqueda (G): 08.VI.2005 1♀, 30.VI.2005 2♀♀; Aramunt (L): 30.VI.2005 1♀.

Analyzed individuals from sampling sites were in accordance with descriptions in Callot *et al.* (1962) and measurements in Delécolle (1985). Some SD intraspecific variation was recorded between individuals: i) SD 3, 9–15 (n = 1), ii) SD 3, 10–15

(n = 1), and iii) SD 3, 11–15. (n = 4). Observed variation was considered intraespecific as all three variants were sequenced and further confirmed to be the same species.

**Known distribution:** Britain Is., Corsica, Danish mainland, French mainland, Germany, Ireland, Northern Ireland, Poland, Portuguese mainland, Slovakia, Spanish mainland, Switzerland, Turkey, Ukraine and Near East.

## *Culicoides gejgelensis* Dzhafarov 1964

**Material.** Canyamars (B): 30.VI.2005 1♀; Susqueda (G): 25.VIII.2005 2♀♀; Alguaire (L): 25.VIII.2005 1♀.

This species belongs to the *Odibilis* group, being closely related to *C. cataneii*. Previous observations reported few morphological differences useful to distinguish *C. cataneii* from *C. gejgelensis*: i) maxillary palp length (Kremer *et al.* 1973), and ii) the antennal ratio (AR), being 1.4–1.5 for *C. cataneii* and 1.2–1.4 for *C. gejgelensis*, (Gil Collado *et al.* 1985). A sequenced *C. cataneii* male allowed the molecular segregation of the females and further definition of morphological diagnostic traits. Our observations (Appendix 1 a-b) did not support the usefulness of these characters, as both values overlapped in sequenced females of both species. Meanwhile differences in the wing pattern were determined: i) presence of macrotrichiae below the basal cell in both species, being distinctively more abundant in *C. gejgelensis*, ii) distal spots on wing margin were more defined in *C. cataneii* (fig. 4). Both species showed few individuals lacking sensillae coeloconica in the 10th antennal segment.

**Known distribution:** Bosnia and Herzegovina, Corsica, Croatia, French mainland, Italian mainland, North Aegean Is., Portuguese mainland, Sicily, Spanish mainland, Turkey, Ukraine, East Palaearctic, Near East and North Africa. Care must be taken when analysing distribution of the species as there would be historical misidentifications of *C. cataneii* and *C. gejgelensis*.

## *Culicoides griseidorsum* Kieffer 1918 (syn. *C. saevanicus* Dzhafarov 1960)

**Material.** Vilanova de la Muga (G): 21.VII. 1♀-1♂, 07.VII.2006 1♀, 27.VII.2006 1♀, 28.VI.2008 1♂, 01.VII.2008 1♀, 03.VII.2008 1♂, 04.VII.2008 1♀, 17.VII.2008 1♂.

This species resembles *C. maritimus*, from which can be morphologically distinguished by: i) antennae, it lacks sensillae coeloconica in the last antennal segment (15th) ii) wing, the cell r2 is partially darkened as a light spot crosses it, whereas in *C. maritimus* the cell is completely darkened (fig. 4), and iii) size, is smaller than *C. maritimus* (Appendices 1–2).

**Known distribution:** Britain Is., Corsica, French mainland, Italian mainland, Poland, Spanish mainland, Near East and North Africa.

## *Culicoides heteroclitus* Kremer & Callot 1965

**Material.** Vilanova de la Muga (G): 22.VI.2006 1♀, 28.IX.2006 1♀; Garcia (T): 02.VI.2005 1♂.

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The characteristic male genitalia was of major importance to confidently identify the species (fig. 3). Moreover, morphological observations of sequenced females were in accordance with descriptions in Callot *et al.* (1965).

**Known distribution:** Dodecanese Is., French mainland, Italian mainland, Portuguese mainland, Spanish mainland and North Africa.

### *Culicoides indistinctus* Khalaf 1961

**Material.** Garcia (T): 18.VIII.2005 1♀, 11.VIII.2005 1♂.

Females of this species fits to *C. odiatus* and *C. lailae* descriptions, however the *C. indistinctus* male examined having a distinctive genitalia, allowed to differentiate the female from the other two species by concordance in the molecular analyses. In addition, all specimens were in accordance with descriptions in Khalaf (1961). According to our results the synonymy of *C. lailae*, and *C. indistinctus* proposed by Remm (1981) should be further revised.

**Known distribution:** according to the synonymy proposed by Remm (1981) and accepted by other authors (Boorman 1989; Dik *et al.* 2006) the distribution of *C. indistinctus* is linked to the distribution of *C. lailae*. Corsica, Crete, Cyprus, Dodecanese Is., French mainland, Greek mainland, Italian mainland, North Aegean Is., Portuguese mainland, Sicily, Spanish mainland, Ukraine, East Palaearctic, Near East and North Africa.

### *Culicoides jumineri* Callot & Kremer 1969

**Material.** Avià (B): 23.VI.2005 1♂, Vilanova de la Muga (G): 04.V.2006 1♂, Aramunt (L): 24.VII.2008 1♂, Amposta (T): 24.V.2007 1♀ 1♂; Vinallop (T): 21.V.2003 1♀.

Morphological analyses were in accordance with descriptions in Kremer *et al.* (1973) for both female and male specimens. Although uniformity was found for most analyzed characters, certain variability was found in female SD. One female had sensillae coeloconica in antennal segment 4, and was lacking in the 12th, whereas another female had sensillae coeloconica in the 6th segment.

**Known distribution:** Corsica, Portuguese mainland, Spanish mainland, Near East and North Africa.

### *Culicoides lailae* Khalaf 1961

**Material.** Aramunt (L): 30.VI.2005 4♀♀, 28.VII.2005 1♂, 29.IX.2005 7♀♀.

The synonymy between *C. lailae* (Khalaf 1961) and *C. odiatus* female (Austen 1921) and male (Edwards 1939) has been widely accepted (Boorman 1989; Glukhova 1989; Rawlings 1996). However differences in thoracic pattern and male genitalia can be detected when comparing original descriptions of both species. Examined individuals had thoracic pattern with spots which was in accordance with *C. lailae* original description (Khalaf 1961), as was the case of the male genitalia. Once molecularly analyzed all the individuals were split into two different groups with genetic distance higher than expected at intraspecific level but lower than expected at interspecific level, being difficult to ascertain if two species were present, whereas no morphological differences were detected between both groups.

**Known distribution:** Is the same than *C. odiatus* as they have been long considered synonym species. Corsica, Crete, Cyprus, Dodecanese Is., French mainland, Greek mainland, Italian mainland, North Aegean Is., Portuguese mainland, Sicily, Spanish mainland, Ukraine, East Palaearctic, Near East and North Africa.

### *Culicoides lupicaris* Downes & Kettle 1952

**Material.** Susqueda (G): 22.VII.2004 7♀♀, 28.X.2004 1♀, 07.XI.2006 1♀, 13.IX.2007 1♀.

The species has been synonymyzed with *C. delta* by Glukhova (1989); however early studies were able to distinguish them by the presence of a dark spot in the cubital wing cell and by thoracic markings (Campbell & Pelhalm-Clinton 1960). Recently, these species have been molecularly differentiated in Gomulski *et al.* (2006). Moreover, *C. pulicaris* is also similar to *C. lupicaris* however the last has a characteristic long dark spot which runs along and in contact with the hind margin of the anal wing cell (Kremer 1965; Delécolle 1985).

**Known distribution:** The known distribution can be biased due to eventual synonymy with *C. delta*. Belarus, Belgium, Britain Is., Corsica, Czech Republic, Danish mainland, Estonia, French mainland, Germany, Ireland, Italian mainland, Lithuania, Northern Ireland, Poland, Romania, Russia, Slovakia, Spanish mainland, Switzerland, Ukraine, East Palaearctic and Near East.

### *Culicoides* (*Monoculicoides*) *parroti* Kieffer 1922

**Material.** Avià (B): 21.VII.2005 1♂; Piera (B): 26.VII.2007 1♀; Caldes de Malavella (G): 12.X.2006 1♀ 1♂; Aramunt (L): 28.VI.2007 1♀.

This species has a characteristic wing pattern with a single dark spot covering most of the radial regions and the distal part of the costa in the upper wing margin (fig. 4), being highly similar to *C. stigma* which was recorded for the first time in the Iberian Peninsula in Goldarazena *et al.* (2008). Females and males of both species can be easily differentiated based on genitalia morphology. All analyzed specimens were in accordance with descriptions in Kremer (1965).

**Known distribution:** Belgium, Britain Is., Corsica, Cyprus, Czech Republic, French mainland, Germany, Hungary, Ireland, Italian mainland, Northern Ireland, Poland, Portuguese mainland, Russia, Sicily, Slovakia, Spanish mainland, Turkey, Ukraine, East Palaearctic, Near East and North Africa.

### *Culicoides* (*Silvaticulicoides*) *picturatus* Kremer & Deduit 1961

**Material.** Canyamars (G): 23.VI.2005 1♀.

The analyzed female was in accordance with descriptions in Kremer *et al.* (1961).

**Known distribution:** Britain Is., Channel Is., Corsica, Danish mainland, French mainland, Italian mainland, Romania, Sicily, Spanish mainland, Turkey, Near East and North Africa.

### *Culicoides poperinghensis* Goetghebuer 1953

**Material.** Avià (B): 02.VI.2005 1♀, 24.V.2007 2♀♀.

A species easy to identify according to the highly characteristic morphology of their sensillae coeloconica, having either 3 or 4 tips, whereas the rest of species had from 5 to 8 tips. In addition all morphological characters analyzed were in accordance with Campbell & Pelhalm-Clinton (1960) and Delécolle (1985).

**Known distribution:** Belgium, Britain Is., Danish mainland, French mainland, Germany, Ireland, Northern Ireland, Romania, Spanish mainland, East Palaearctic, Near East and North Africa.

### Culicoides pseudopallidus Khalaf 1961

**Material.** Alguaire (L): 29.IX.2005 3♀♀-1♂, 19.VII.2007 1♂, 26.VII.2007 1♂; Aramunt (L): 14.VII.2005 1♂; Garcia (T): 07.VII.2005 1♀, 28.VII.2005 1♂.

All female and male specimens were in accordance with descriptions in Khalaf (1961) and Kremer et al. (1973).

**Known distribution:** French mainland, Portuguese mainland, Near East and North Africa.

### Culicoides (Monoculicoides) puncticollis (Becker 1903)

**Material.** Vilanova de la Muga (G): 21.VII.2005 1♀.

The female analyzed was in accordance with Kremer (1965).

**Known distribution:** Balearic Is., Belgium, Britain Is., Corsica, Cyprus, Dodecanese Is., French mainland, Hungary, Italian mainland, Poland, Portuguese mainland, Slovakia, Spanish mainland, Turkey, Ukraine, East Palaearctic, Near East and North Africa.

### Culicoides (Oecacta) sahariensis Kieffer 1923

**Material.** Garcia (T): 14.VII.2005 1♀; Aramunt (L): 10.VIII.2006 1♂.

This species belongs to the *Similis* group, and is similar to *C. coluzzii* and *C. yemenensis* according to wing pattern and morphological measurements. However, females of these species can be differentiated using wing pattern and SD. The light spot from m1 did not cross veins M1 and M2 neither in *C. sahariensis* nor in *C. yemenensis*, whereas in *C. coluzzii* did. Although wing pattern was extremely similar for *C. sahariensis* and *C. yemenensis*, the SD (Appendix 1a) proved useful to distinguish the females and resulting groups were supported by sequencing results.

In addition, male genitalia was useful to easily distinguish these species. Aedeagus shape in *C. yemenensis* is quite different than in C. sahariensis (fig. 3) and *C. coluzzii*, the later two being distinguishable by the shape of ventral apodema, simple shape in *C. sahariensis*, and chair shape in *C. coluzzii*. (Callot et al. 1970; Kremer 1965). Morphological measurements were in accordance with those present in Boorman (1989).

**Known distribution:** Cyprus, Italian mainland, Portuguese mainland, Sicily, Spanish mainland, Turkey, Near East and North Africa.

### Culicoides (Pontoculicoides) sejfadinei Dzhafarov 1958

**Material.** Aramunt (L): 09.VI.2005 2♀♀.

Reexamination of these two females, being previously identified as *C. saevus* (Sarto i Monteys et al. 2009), allowed us to identify them as *C. sejfadinei* based on SD. *Culicoides sejfadinei* has sensillae coeloconica in antennal segments 3, and from 5 until 10, which is useful to differentiate it from *C. saevus* with SD in segments 3 and from 7 until 10, despite having the two species three spermathecae of similar shape (fig. 3). The remaining analyzed characters were in accordance with those described in Glukhova (1989).

**Known distribution:** Bosnia and Herzegovina, Dodecanese Is., French mainland, Greek mainland, Italian mainland, Sicily, East Palaearctic and Near East.

### Culicoides simulator Edwards 1939

**Material.** Susqueda (G): 30.VI.2005 1♀, 07.VII.2005 1♀.

Both females analyzed were in accordance with descriptions in Kremer (1965).

**Known distribution:** Belarus, Belgium, Britain Is., Channel Is., Czech Republic, Danish mainland, Estonia, Germany, Hungary, Italian mainland, Lithuania, Poland, Russia, Slovakia, Spanish mainland, Turkey, Ukraine, East Palaearctic, Near East and North Africa.

### Culicoides submaritimus Dzhafarov 1962

**Material.** Vilanova de la Muga (G): 26.VI.2008 1♀ 1♂, 03.VII.2008 2♂♂.

*Culicoides submaritimus* has been long considered as a synonym of *C. maritimus* by several authors (Glukhova 1989; Rawlings 1996; Dik et al. 2006). All specimens morphologically analyzed were previously sequenced and confirmed to be the same species but not *C. maritimus*. Morphological differences useful to distinguish both *C. submaritimus* and *C. maritimus* males and females were found, being the most significant: i) the absence of sensillae coeloconica in antennal segment 15$^{th}$ in *C. submaritimus*, and ii) the bigger size of the r5 light spot in *C. submaritimus* (fig. 4) (Kremer et al. 1973).

**Known distribution:** As a result of the synonymy with *C. maritimus*, the distribution of both species is overlapped, being difficult to infer their separate distributions. Belgium, Britain Is., Corsica, Cyprus, Czech Republic, Danish mainland, French mainland, Germany, Hungary, Italian mainland, Poland, Portuguese mainland, Romania, Russia, Sicily, Slovakia, Spanish mainland, Ukraine, East Palaearctic, Near East and North Africa.

### Culicoides (Oecacta) truncorum Edwards 1939 (syn. C. sylvarum Boorman 1984)

**Material.** Canyamars (B): 30.VI.2005 1♀.

The analyzed female was in accordance with Boorman (1984) descriptions. *Culicoides truncorum* has been synonymyzed with *C. sylvarum* by Boorman (1984).

**Known distribution:** Belgium, Britain Is., Czech Republic, Danish mainland, Estonia, French mainland, Germany, Russia, Slovakia, Spanish mainland, Turkey, Ukraine, East Palaearctic, Near East and North Africa.

### Culicoides (Oecacta) vexans (Stæger 1839)

**Material.** Vilanova de la Muga (G): 03.VI.2004 1♀.

The analyzed female was in accordance with descriptions in Kremer (1965), however length of the antenna was bigger than expected and wing spots were not appreciated until wing digital pictures were contrasted.

**Known distribution:** Austria, Belgium, Britain Is., Danish mainland, Estonia, Finland, French mainland, Germany, Hungary, Ireland, Lithuania, Northern Ireland, Norwegian mainland, Poland, Portuguese mainland, Russia, Slovakia, Spanish mainland, Turkey, Ukraine, East Palaearctic and Near East.

### Culicoides yemenensis Boorman 1989

**Material.** Alguaire (L): 01.IX.2005 1♀, 26.VI.2008 1♀, Aramunt (L): 01.IX.2005 1♀, 10.VIII.2006 1♂.

This species belong to the *Similis* group, where similar wing patterns are found for other species in the group: *C. coluzzii*, *C. sahariensis* and *C. similis*. Specimens of *C. yemenensis* can be easily distinguished by: i) the characteristic male genitalia of *C. yemenensis*, and ii) the distribution of sensillae coeloconica (SD) through the antennae in female specimens. *Culicoides yemenensis* had sensillae in all short antennal segments (3-10) whereas in the other mentioned species SD is 3, 5, 7-10, although Boorman (1989) reported some *C. sahariensis* female specimens of difficult identification for having sensillae coeloconica in antennal segments 4 and 6.

Morphological observations of all diagnosed individuals were in agreement with descriptions in Boorman (1989). The only exception was the spot lying close to the r2 which was similar to that found for *C. sahariensis*, not for *C. yemenensis* (fig. 4). Nevertheless observations of the differential male genitalia (fig. 3), combined with the molecular analysis of both *C. yememensis* and *C. sahariensis* male and female individuals, demonstrate that these were close but differentiated species and both were present in the analyzed area.

Reexamination of two specimens (Aramunt: 01.IX.2005, 10.VIII.2006), being previously identified as *C. sahariensis* (Sarto i Monteys *et al.* 2009), allowed us to identify them as *C. yemenensis* based on the distribution of sensillae coeloconica (SD) and the molecular studies.

**Known distribution:** Yemen.

### Notes on regional distribution of *Culicoides* species

Overall within all the species found, those previously reported by our research group in Ventura *et al.* (2005) have not been described above, however geographic distribution of species among the four provinces was assessed and divided into three different categories:

i) The first group was composed of species that were only found within the same province in the current survey as well as in the former one (Ventura *et al.* 2005): *C. alazanicus*, *C. univittatus* and *C. subfagineus* recorded in Girona province.

ii) Species in the second group were those that were found in the same provinces than in the former survey and in new ones: *C. achrayi*, *C. kibunensis* and *C. cataneii* were all new records from Girona province. *Culicoides begueti* was found in Barcelona and Lleida provinces. *Culicoides circumscriptus*, *C. newsteadi* s.l. and *C. imicola* showed a new distribution within Lleida province in sampling sites with continental climate under which *C. imicola* was not expected to be found. *Culicoides obsoletus*, *C. pulicaris* s.l., and *C. punctatus* were new records for Lleida and Tarragona provinces whereas *C. flavipulicaris* and *C. scoticus* for Girona, Lleida and Tarragona provinces.

iii) The third group of species includes species which have not been found in some of the provinces where were reported in 2005: *C. kurensis* was previously found in Girona and Tarragona provinces but now only in Tarragona and Lleida, *C. maritimus* was previously
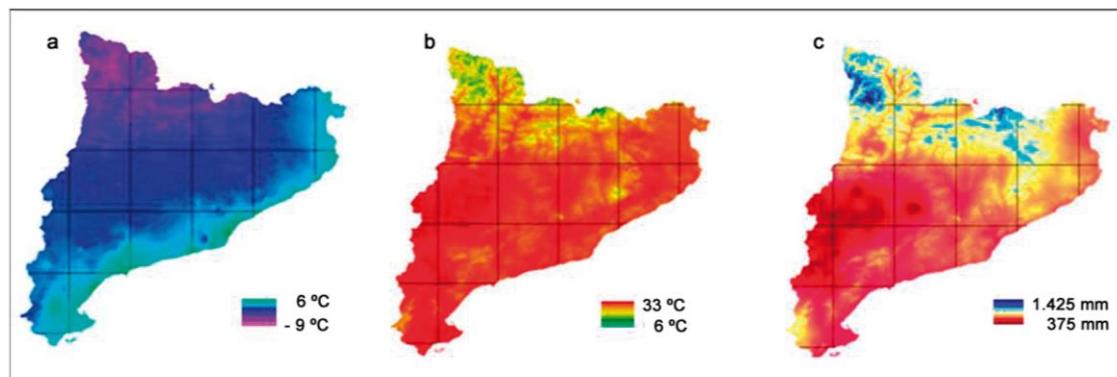


**Figure 5**
Raster maps of Catalonia for **a,** mean low temperature of January; **b,** mean high temperature of July, and **c,** annual precipitation rate.

found in Barcelona, Girona and Tarragona provinces and now only in Tarragona, its absence from Girona province could be the result of a wrong morphological identification confusing it with *C. submaritimus* or *C. griseidorsum*, both morphologically similar and present there. Finally *C. pallidicornis* and *C. subfascipennis* were previously found in Barcelona province but now only in Girona.

There is a group of species from the previous work of Ventura *et al.* (2005) which have not been found, some of them previously detected in sampling sites not used (*C. deltus*, *C. fascipennis*, *C. impunctatus*, *C. minutissimus* and *C. odiatus*), whereas others were found in sampling sites used in the present study (*C. longipennis* and *C. pictipennis*). In contrast, we found several new recorded species in three sampling sites previously
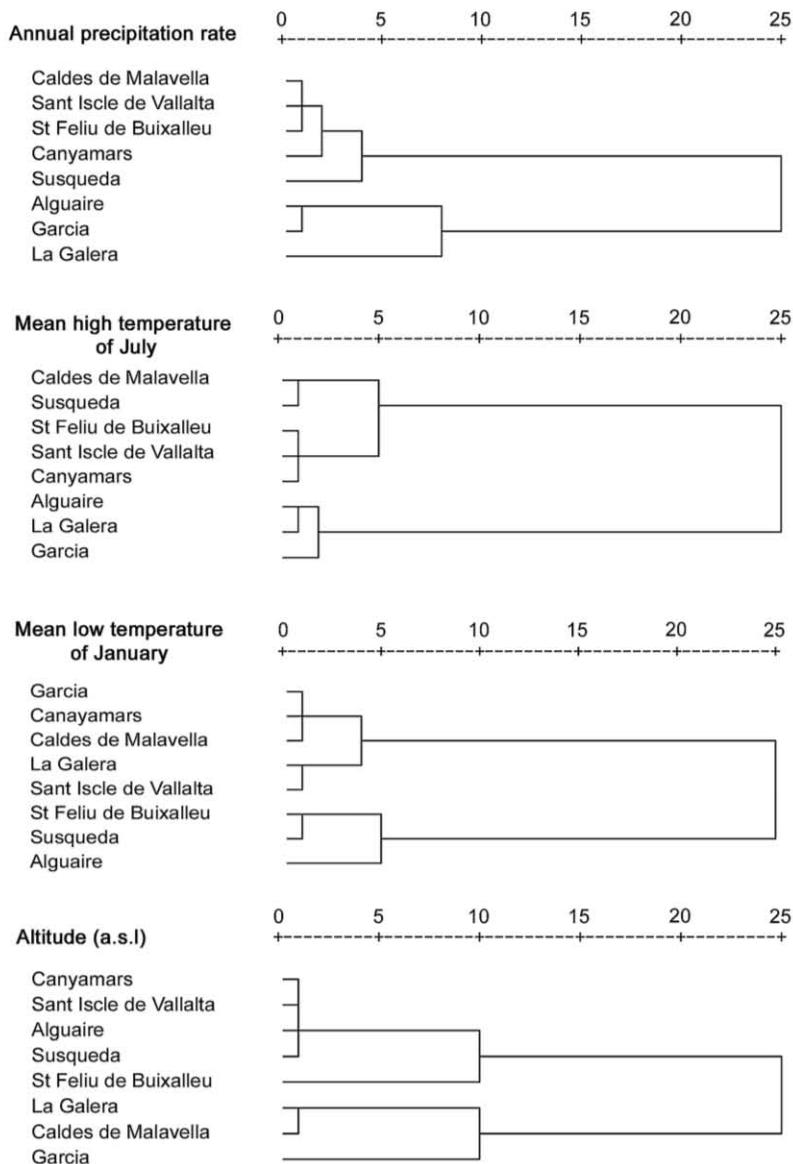


**Figure 6**
Dendrograms generated with the bioclimatic variables, using average linkage (betwen groups).

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used: i) in Vilanova de la Muga new species recorded were *C. circumscriptus*, *C. festivipennis*, *C. flavipulicaris*, *C. griseidorsum*, *C. heteroclitus*, *C. jumineri*, *C. newsteadi* s.l., *C. obsoletus*, *C. pulicaris* s.l., *C. punctatus*, *C. puncticollis*, *C. scoticus*, *C. subfagineus*, *C. submaritimus*, and *C. vexans* ii) in La Galera were *C. circumscriptus*, *C. coluzzii*, *C. newsteadi* s.l., *C. obsoletus*, *C. punctatus* and *C. scoticus* , and iii) in Vinallop was *C. jumineri*.

The increase in both the number of *Culicoides* species recorded and the distribution for some of these species, when compared with Ventura *et al.* (2005), was the result of the addition of new sampling sites combined with the replacement of some of the old ones, the detection of non previously reported scarce species, and the improvement of species morphological identification when combined with sequencing results that allowed a confident identification for all species, even cryptic ones.

Regarding the influence of bioclimatic variables on *Culicoides* distribution, once generated the four dendrograms for bioclimatic variables (fig. 6), two of them grouped localities in two clusters which were coincident with geographic distribution of *Culicoides* species from Group 1 (*C. achrayi*, *C. brunnicans*, *C. dewulfi*, *C. furcillatus* and *C. lupicaris*) and Group 2 (*C. coluzzii*, *C. indistinctus*, *C. lailae*, *C. pseudopallidus* and *C. sahariensis*). These two bioclimatic variables were mean high temperature of July (the warmest month) and annual precipitation rate. Thus suggesting that these were the only variables analyzed influencing the geographical distribution of both groups of species in the surveillance area.

In locations where species from Group 1 were present and those from Group 2 absent, the mean high temperature of July ranged from 27.5 °C to 29.6 °C and annual precipitation rate ranged from 739.0 mm to 916.8 mm. In contrast, those locations with species from Group 2 present and those from Group 1 absent, values ranged from 31.9 °C to 32.8 °C and from 427.3 mm to 445.2 mm respectively. Interestingly in Aramunt, the only location where species of both groups were present, both variables showed intermediate values, 30.6 °C and 678.2 mm.

## Conclusions

Overall, the present study allowed a sustantial increase in the number of species recorded in the area, from 26 species detected in our last revision (Ventura *et al.* 2005) and 40 in recent work by Sarto *et al.* (2009) to 53 identified to date. In addittion, this lead to an improvement of their distribution knowledge. This highlights that fast species identification mainly based on wing morphology, which is widely used in surveillance

programmes, or detailed analysis of only some of the specimens can lead to species misidentification and consequently to an underestimation of the area's *Culicoides* diversity. In fact, the accurate reexamination of 5 out of the 88 specimens identified as *C. sahariensis* in Sarto i Monteys *et al.* (2009) resulted in the detection of 2 *C. yemenensis* (Aramunt: 01-IX- 2005 and 10-VIII-2006) being a new record for Europe, as well as the detection of 1 *C. coluzzii* (La Galera: 01-IX-2005) being a new record of the Iberian Peninsula.

The accurate study combining both morphlgical identification techniques and molecular analyses, allowed a reliable identification of all individuals, even those belonging to a group of morphologically similar species. In consequence we suggest that this type of studies should be implemented in those areas where a deep knowledge of *Culicoides* diversity is required.

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## On line appendices:

Appendix 1a. Female antennae metric characters recorded from all *Culicoides* species identified.
Appendix 1b. Female palp and wing metric characters, and number of spermathecae recorded from all *Culicoides* species identified.
Appendix 2a. Male antennae metric characters recorded from all *Culicoides* species identified.
Appendix 2b. Male palp and wing metric characters recorded from all *Culicoides* species identified.

# Appendices

**Appendix 1a.** Female antennae metric characters recorded from all *Culicoides* species identified
AR, ratio of the sum of the lengths of the apical five segments of the flagellum (11–15) to the sum of the basal eight (3–10); SD, sensillae coeloconica distribution among the segments of the flagellum; R11/10, length of the 11th segment of the flagellum to the 10th; AL, sum of the lengths from the 3rd segment of the flagellum to the 15th.

| | n | AR | SD | R 11/10 | AL(μm) |
|---|---|---|---|---|---|
| *C. achrayi* | 2 | 1.12–1.13 | 3, 10–15 | 1.50–1.56 | 627.5–715 |
| *C. alazanicus* | 1 | 1.67 | 3–15 | 2.28 | 755 |
| *C. begueti* | 1 | 1.13 | 3–9, 11–15 | 1.53 | 612.5 |
| *C. brunnicans* | 1 | 1.17 | 3, 5, 7–9, 11–15 | 1.4 | 590 |
| *C. cataneii* | 7 | 1.24–1.41 | 3–9, 11–15 | 1.58–1.92 | 473–620 |
| *C. circumscriptus* | 1 | 1.44 | 3–14 | 1.78 | 652.5 |
| *C. coluzzii* | 3 | 1.18–1.29 | 3, (4), 5, 7–10 | 1.50–1.55 | 445–487 |
| *C. dewulfi* | 1 | 1.19 | 3, 11–15 | 1.36 | 542.5 |
| *C. fagineus s.l.* | 8 | 0.82–1.11 | 3, 11–15 | 1.13–1.45 | 295–360 |
| *C. festivipennis* | 2 | 1.39 | 3–15 | 1.69–1.78 | 545–687.5 |
| *C. flavipulicaris* | 2 | 1.19 | 3, 11–15 | 1.43–1.53 | 735 |
| *C. furcillatus* | 6 | 1.01–1.09 | 3, 9–15 | 1.33–1.44 | 562.5–675 |
| *C. gejgelensis* | 4 | 1.20–1.28 | 3–9, 11–15 | 1.50–1.73 | 502.5–570 |
| *C. griseidorsum* | 5 | 1.07–1.23 | 3–14 | 1.47–1.64 | 527.5–620 |
| *C. heteroclitus* | 2 | 1.13–1.19 | 3–15 | 1.50–1.54 | 537.5–570 |
| *C. imicola* | 4 | 1.14 | 3, 11–15 | 1.38–1.43 | 497.5–545 |
| *C. indistinctus* | 1 | 1.2 | 3–14 | 1.5 | 577.5 |
| *C. jumineri* | 2 | 1.18–1.28 | 3, 5, 7, 9, 11–15 | 1.27–1.69 | 550–595 |
| *C. kibunensis* | 1 | 1.54 | 3–15 | 2.11 | 470 |
| *C. kurensis* | 3 | 1.08–1.19 | 3, 7–14 | 1.25–1.61 | 497.5–550 |
| *C. lailae* | 11 | 0.99–1.18 | 3–(10) 14 | 1.25–1.46 | 452.5–577.5 |
| *C. lupicaris* | 10 | 0.98–1.14 | 3, 11–15 | 1.29–1.61 | 707.5–932.5 |
| *C. maritimus* | 1 | 1.36 | 3–15 | 1.88 | 790 |
| *C. newsteadi s.l.* | 32 | 0.69–1.07 | 3, 11–15 | 1.23–1.47 | 642.5–877.5 |
| *C. obsoletus* | 5 | 1.08–1.16 | 3, 11–15 | 1.27–1.38 | 542.5–620 |
| *C. pallidicornis* | 2 | 1.13 | 3, 11–15 | 1.33–1.45 | 457.5–490 |
| *C. parroti* | 3 | 0.95 | 3, 8–10 | 1.28 | 737.5 |
| *C. picturatus* | 1 | 1.02 | 3, 11–15 | 1.33 | 570 |
| *C. poperinghensis* | 3 | 1.18–1.24 | 3 ,(9),(10), 11–15 | 1.47–1.50 | 617.5–635 |
| *C. pseudopallidus* | 4 | 1.27–1.31 | 3–15 | 1.55–1.58 | 505–537.5 |
| *C. pulicaris s.l.* | 37 | 0.91–1.37 | 3, 11–15 | 1.29–1.61 | 667.5–932.5 |
| *C. punctatus* | 2 | 1.09–1.10 | 3, 11–15 | 1.40–1.53 | 652.5–772.5 |
| *C. puncticollis* | 1 | 0.76 | 3, 8–10 | 1.07 | 560 |
| *C. sahariensis* | 1 | 1.3 | 3, 5, 7–10 | 1.55 | 477.5 |
| *C. scoticus* | 2 | 1.17–1.23 | 3, 11–15 | 1.17–1.47 | 547.5–625 |
| *C. sejfadinei* | 2 | 1.04–1.06 | 3, 5–10 | 1.15–1.18 | 432.5–500 |
| *C. simulator* | 2 | 1.22–1.36 | 3, 5, 7–10 | 1.61–1.64 | 560–607.5 |
| *C. subfagineus* | 6 | 0.87–0.93 | 3, 11–15 | 1.21–1.39 | 687.5–745 |
| *C. subfasciipennis* | 3 | 1.08–1.11 | 3, 11–15 | 1.46–1.53 | 547.5–610 |
| *C. submaritimus* | 1 | 1.43 | 3–14 | 1.81 | 752.5 |
| *C. truncorum* | 1 | 1.25 | 3, 5, 7, 9, 11–15 | 1.67 | 545 |
| *C. univittatus* | 1 | 1.33 | 3–15 | 1.85 | 885 |
| *C. vexans* | 1 | 1.14 | 3, 5, 7, 9, 11–15 | 1.44 | 787.5 |
| *C. yemenensis* | 3 | 1.20–1.32 | 3–10 | 1.25–1.46 | 462.5–527.5 |

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**Appendix 1b.** Female palp and wing metric characters, and number of spermathecae recorded from all *Culicoides* species identified.
PR, ratio of the length of the 3rd palpal segment to the sum of the lengths of the 1st and 2nd; PL, sum of the lengths of the five palpal segments; WL, wing length measured from arculus to apex; CL, costa length measured from arculus; WB, wing breadth measured at its widest point; SN, spermathecae number.

|  | PR | PL(μm) | WL(mm) | CL(mm) | WB(mm) | SN |
|---|---|---|---|---|---|---|
| C. achrayi | 0.68–0.92 | 237.5–245 | 1.30–1.42 | 0.80–0.87 | 0.62–0.63 | 2 |
| C. alazanicus | 1.43 | 207.5 | 1.35 | 0.88 | 0.63 | 2 |
| C. begueti | 1.4 | 167.5 | 1 | 0.6 | 0.48 | 2 |
| C. brunnicans | 0.87 | 247.5 | 1.33 | 0.82 | 0.65 | 2 |
| C. cataneii | 0.96–1.26 | 165–195 | 0.95–1.23 | 0.53–0.72 | 0.45–0.58 | 2 |
| C. circumscriptus | 0.89 | 230 | 1.38 | 0.78 | 0.6 | 1 |
| C. coluzzii | 1.10–1.31 | 145–160 | 0.95–1.05 | 0.50–0.55 | 0.45–0.57 | 2 |
| C. dewulfi | 1 | 185 | 1.18 | 0.73 | 0.58 | 2 |
| C. fagineus s.l. | 0.91–1.48 | 103–300 | 1.25–1.50 | 0.78–0.98 | 0.58–0.68 | 2 |
| C. festivipennis | 0.85–0.87 | 192.5–250 | 1.37 | 0.82 | 0.62 | 2 |
| C. flavipulicaris | 1 | 247.5 | 1.23–1.38 | 0.68–0.87 | 0.55–0.60 | 2 |
| C. furcillatus | 0.73–0.78 | 192.5–230 | 1.05–1.30 | 0.65–0.77 | 0.55–0.65 | 2 |
| C. gejgelensis | 1.08–1.32 | 180–205 | 1.00–1.15 | 0.53–0.68 | 0.50–0.53 | 2 |
| C. griseidorsum | 0.89–1.04 | 195–227.5 | 1.00–1.23 | 0.55–0.73 | 0.48–0.55 | 2 |
| C. heteroclitus | 0.97–1.00 | 205 | 1.05–1.10 | 0.60–0.63 | 0.48–0.50 | 2 |
| C. imicola | 0.68–0.96 | 172.5–195 | 1.00–1.13 | 0.53–0.63 | 0.50–0.58 | 2 |
| C. indistinctus | 1.03 | 230 | 1.03 | 0.55 | 0.5 | 2 |
| C. jumineri | 1.07–1.19 | 202.5–210 | 1.08–1.20 | 0.60–0.68 | 0.52–0.57 | 2 |
| C. kibunensis | 1.28 | 142.5 | – | – | 0.4 | 2 |
| C. kurensis | 0.97–1.00 | 182.5–202.5 | 1.03–1.08 | 0.60–0.63 | 0.50–0.53 | 2 |
| C. lailae | 0.81–0.97 | 157.5–210 | 0.98–1.25 | 0.55–0.75 | 0.45–0.60 | 2 |
| C. lupicaris | 0.62–0.78 | 232.5–315 | 1.35–1.68 | 0.83–1.03 | 0.58–0.75 | 2 |
| C. maritimus | 1 | 277.5 | 1.82 | 1.05 | 0.75 | 2 |
| C. newsteadi s.l. | 0.73–1.12 | 210–315 | 1.20–1.90 | 0.65–1.13 | 0.55–0.83 | 2 |
| C. obsoletus | 0.64–0.88 | 162.5–192.5 | 1.08–1.18 | 0.63–0.73 | 0.50–0.53 | 2 |
| C. pallidicornis | 0.61–0.68 | 157.5–167.5 | 0.88–0.93 | 0.53 | 0.43–0.48 | 2 |
| C. parroti | 0.92–1.09 | 272.5–342.5 | 1.13–1.60 | 0.63–0.85 | 0.65–0.73 | 1 |
| C. picturatus | 0.84 | 197.5 | 1.13 | 0.65 | 0.53 | 2 |
| C. poperinghensis | 0.77–0.85 | 222.5 | 1.24–1.33 | 0.73–0.78 | 0.60–0.63 | 2 |
| C. pseudopallidus | 1.04–1.11 | 195–212.5 | 1.00–1.13 | 0.6 | 0.48–0.55 | 2 |
| C. pulicaris s.l. | 0.58–0.93 | 232.5–315 | 1.23–1.88 | 0.73–1.03 | 0.58–0.78 | 2 |
| C. punctatus | 0.94 | – | 1.25–1.50 | 0.75–0.88 | 0.58–0.70 | 2 |
| C. puncticollis | 0.92 | 275 | 1.45 | 0.75 | 0.63 | 1 |
| C. sahariensis | 1.16 | 147.5 | 0.9 | 0.5 | 0.45 | 2 |
| C. scoticus | 0.65–0.73 | 180–202.5 | 1.10–1.28 | 0.70–0.78 | 0.55–0.60 | 2 |
| C. sejfadinei | 0.87–1.09 | 172.5–200 | 0.98–1.07 | 0.53–0.58 | 0.47–0.55 | 3 |
| C. simulator | 1.06–1.07 | 207.5–222.5 | 1.13–1.23 | 0.63–0.70 | 0.48–0.53 | 2 |
| C. subfagineus | 1.03–1.19 | 207.5–262.5 | 0.98–1.20 | 0.58–0.75 | 0.45–0.55 | 2 |
| C. subfasciipennis | 0.83 | 190–202.5 | 1.13–1.25 | 0.67–0.73 | 0.55–0.60 | 2 |
| C. submaritimus | 1.05 | 277.5 | 1.45 | 0.83 | 0.65 | 2 |
| C. truncorum | 0.92 | 172.5 | 0.98 | 0.58 | 0.45 | 2 |
| C. univittatus | 1.07 | – | 1.95 | 1.23 | 0.85 | 2 |
| C. vexans | 1.02 | 312.5 | 1.75 | 1 | 0.8 | 2 |
| C. yemenensis | 1.32–1.38 | 155 | 0.92–1.00 | 0.50–0.60 | 0.47–0.50 | 2 |

2

New insights of *Culicoides* from NE Spain

**Appendix 2a.** Male antennae metric characters recorded from all *Culicoides* species identified.

AR, ratio of the sum of the lengths of the apical three segments of the flagellum (13-15) to the sum of the basal ten (3-12); SD, sensillae coeloconica distribution among the segments of the flagellum; R13/12, length of the 13th segment of the flagellum to the 12th; AL, sum of the lengths from the 3rd segment of the flagellum to the 15th.

|  | n | AR | SD | R13/12 | AL(μm) |
|---|---|---|---|---|---|
| C. alazanicus | 3 | 0.88 | 3, 13–15 | 3.06–3.76 | 682.5 |
| C. begueti | 2 | – | – | – | – |
| C. cataneii | 1 | 0.8 | 3, 13–15 | 3 | 630 |
| C. derisor | 1 | – | – | 2.83 | – |
| C. dewulfi | 1 | – | 3, 13–15 | 2.69 | – |
| C. griseidorsum | 4 | 0.67–0.69 | 3, 10, 13, 14 | 2.60–2.83 | 765–795 |
| C. heteroclitus | 1 | – | 3, 13–15 | 2.93 | – |
| C. indistinctus | 1 | – | – | – | – |
| C. jumineri | 4 | 0.72–0.85 | 3, 13–15 | 2.77–3.21 | 585–680 |
| C. kibunensis | 3 | 0.78–0.80 | 3, 13–15 | 2.71–3.08 | 575–577.5 |
| C. lailae | 1 | – | – | – | – |
| C. maritimus | 3 | 0.73–0.75 | 3, 10, 13–15 | 3.00–3.75 | 775–902.5 |
| C. newsteadi s.l. | 15 | 0.61–0.69 | 3, 13–15 | 1.82–2.67 | 792.5–890 |
| C. obsoletus | 1 | – | – | – | – |
| C. parroti | 2 | – | – | – | – |
| C. pseudopallidus | 5 | – | – | – | – |
| C. pulicaris s.l. | 2 | 0.61 | 3, 13–15 | 1.96–2.07 | 995–998.5 |
| C. punctatus | 1 | – | – | – | – |
| C. sahariensis | 1 | 0.63 | 3, 8–12 | 2.25 | 510 |
| C. scoticus | 9 | 0.71 | 3, 13–15 | 2.75–3.10 | 930 |
| C. submaritimus | 3 | 0.75 | 3, 10, 13, 14 | 2.68–3.05 | 809.5 |
| C. yemenensis | 1 | – | – | – | – |

**Appendix 2b.** Male palp and wing metric characters recorded from all *Culicoides* species identified.

PR, ratio of the length of the 3rd palpal segment to the sum of the lengths of the 1st and 2nd; PL, sum of the lengths of the five palpal segments; WL, wing length measured from arculus to apex; CL, costa length measured from arculus; WB, wing breadth measured at its widest point.

|  | PR | PL(μm) | WL(mm) | CL(mm) | WB(mm) |
|---|---|---|---|---|---|
| C. alazanicus | 1.00–1.18 | 140–147.5 | 0.98–1.05 | 0.53–0.55 | 0.35–0.37 |
| C. begueti | 1.06–1.13 | 127.5–130 | 0.75–0.82 | 0.38–0.40 | 0.30–0.35 |
| C. cataneii | 1 | 130 | 1.05 | 0.53 | 0.4 |
| C. derisor | 0.8 | 140 | 0.78 | 0.38 | 0.3 |
| C. dewulfi | 0.83 | 165 | 1.25 | 0.73 | 0.47 |
| C. griseidorsum | 1 | 165–180 | 1.10–1.17 | 0.57–0.63 | 0.37–0.40 |
| C. heteroclitus | 1.19 | 177.5 | 1.03 | 0.53 | 0.4 |
| C. indistinctus | 1 | 172.5 | 0.93 | 0.47 | 0.38 |
| C. jumineri | 1.00–1.10 | 147.5–167.5 | 0.85–1.08 | 0.43–0.58 | 0.35–0.43 |
| C. kibunensis | 1.00–1.42 | 122.5–130 | 0.83 | 0.40–0.43 | 0.33 |
| C. lailae | – | – | – | – | – |
| C. maritimus | 1.00–1.04 | 162.5–202.5 | 1.13–1.43 | 0.54–0.75 | 0.40–0.53 |
| C. newsteadi s.l. | 0.88–1.14 | 177.5–215 | 1.10–1.35 | 0.58–0.73 | 0.40–0.48 |
| C. obsoletus | 0.9 | 162.5 | 1.15 | 0.7 | 0.43 |
| C. parroti | 0.92–0.95 | 190–205 | 1.15–1.25 | 0.55–0.63 | 0.45–0.55 |
| C. pseudopallidus | 0.94–1.09 | 147.5–187.5 | 1.05–1.08 | 0.50–0.55 | 0.40–0.43 |
| C. pulicaris s.l. | 0.87–0.92 | 237.5–245 | 1.57–1.70 | 0.77–1.00 | 0.55–0.57 |
| C. punctatus | 0.92 | 187.5 | 1.35 | 0.78 | 0.5 |
| C. sahariensis | 1 | 107.5 | 0.78 | 0.35 | 0.35 |
| C. scoticus | 0.73–0.96 | 160–187.5 | 1.15–1.53 | 0.68–0.93 | 0.43–0.55 |
| C. submaritimus | 0.96–1.04 | 190–197.5 | 1.18–1.20 | 0.63–0.65 | 0.45 |
| C. yemenensis | 1.08 | 117.5 | 0.78 | 0.35 | – |

3

**Capítol II**

## Abstract

**Background:** The genus *Culicoides* (Diptera: Ceratopogonidae) is well known for their importance in the field of medical and veterinary entomology. *Culicoides* transmit a large variety of pathogens, primarily viruses that affect animals and humans. In Europe, the most economically important disease transmitted by *Culicoides* is Bluetongue (BT). *Culicoides* have been recently involved as primary vectors for Schmallenberg disease.

The taxonomy within the subgenus *Culicoides* has been historically difficult and reorganizations have been proposed regularly. The subgenus *Culicoides* has species considered potential vectors for BT. High morphological intraspecific variability has been attributed to these species. This fact highlighted the apparent presence of previously undetected cryptic species diversity in the subgenus. In the present study, a concise analysis of morphological traits and DNA sequencing was performed to specimens from the Pulicaris or Punctatus groups.

**Methods:** *Culicoides* specimens described were trapped in Spain between years 2004-2009 using CDC black light traps. Biting midges in the Pulicaris or Punctatus groups were selected and each individual was analyzed through morphology and sequencing. Morphological analyses were based on 16 traits for females and 14 for males. Sequencing was based on a fragment of the subunit I of the cytochrome oxidase (COI) gene. DNA sequences were used to define interspecific boundaries for some morphological traits.

**Results:** The detailed morphological and molecular study of specimens belonging to *Culicoides pulicaris* s.l. and specimens resembling a cross between *C. pulicaris* and *C. punctatus*, revealed the presence of two new species: *C. cryptipulicaris* and *C. quasipulicaris.* Females of *C. quasipulicaris* and males of both species could be morphologically distinguished from *C. pulicaris* (Linnaeus, 1758) whereas females of *C. cryptipulicaris* could be identified exclusively using molecular techniques. A new morphological dicothomic key is provided for the identification of adult males and females of species resembling *C. pulicaris* and *C. puntcatus* worldwide*.*

**Conclusion:** Combining traditional morphological approaches with molecular barcoding techniques two new species of *Culicoides* have been described, *C. cryptipulicaris* and *C. quasipulicaris*. The new species described belong to the subgenus *Culicoides* and are

phylogenetically closely related to other species considered potential vectors of diseases in Europe.

K**eywords:** *Culicoides cryptipulicaris*, *Culicoides quasipulicaris*, new species, , cryptic species,DNA barcoding, morphology, dicotomic key, Bluetongue, Schmallenberg

## Background

The genus *Culicoides* (Diptera: Ceratopogonidae) is currently composed of more than 1400 species. Most of them have been properly described and classified among 30 subgenera, although 497 species still remain in unplaced subgenus [1]. *Culicoides* midges are well known for their importance in the field of medical and veterinary entomology. They transmit a large variety of pathogens, primarily viruses (but protozoa and filarial worms as well) that affect humans and animals [2]. In Europe, the most economically important disease transmitted by *Culicoides* is Bluetongue (BT). Since the re-emergence of BT in Europe (1999) [3], the viral disease has been circulating all over EU territories, sometimes with unpredictable dynamics. Nowadays, we are witnessing the endemicity of this economically devastating disease that can severely affect domestic and wild ruminants and is of mandatory declaration to OIE. Besides BT, *Culicoides* have a major epidemiological role as well in the recently discovered Schmallenberg (SB) disease in Europe [4].

The taxonomical work we address is focused on the description of new cryptic species within the subgenus *Culicoides*. To epidemiologically focus that work, it is relevant to note that, in Europe, BT outbreaks unexpectedly occurred in regions beyond the known distribution range of *C. imicola* (main afro-asiatic BT vector). The most remarkable case was the BTV-8 epizooty appeared in central Europe (2006; [3]). Entomological studies in affected areas beyond *C. imicola* range led to incriminate other Palaearctic authochtonous *Culicoides* as BT vectors [5-12]. Since then, the *Culicoides* species considered as potential vectors for BT belonged to the subgenera *Avaritia* (*C. obsoletus, C. scoticus, C. dewulfi, and C. chiopterus*) and Culicoides (*C. pulicaris, C. punctatus,* and *C. newsteadi*). Beyond Europe, the subgenus *Culicoides* has been related with livestock diseases, being the case of *C. magnus* Colaco, 1946 for BT disease [13]. Moreover, species in the subgenus *Culicoides* have been related with SB virus in outbreak areas in Europe [4,14].

The taxonomy within the subgenus *Culicoides* has been historically difficult and has experienced constant reorganizations [15-22]. Molecular techniques have clarified (partly) the current situation in that subgenus. The subgenus *Culicoides* showed to have species with high intraspecific variability. This fact highlighted the apparent presence of previously undetected cryptic species diversity in the subgenus [13, 23-29].

Species in the subgenus *Culicoides* have a characteristic r5 spot hourglass-shaped at the wing that can be more or less defined [19]. Wing diagnostic characters to separate Pulicaris and Punctatus species groups from other species within the subgenus are: i) presence of a dark spot in cubital wing cel, and ii) a dark spot point- shaped proximaly on second medial wing vein. Worldwide, there are 10 described species with the former described characters attibuted to Pulicaris and Punctatus species groups (Table 1). Five species resemble *C. pulicaris*, the authors grouped them in the Pulicaris group (*C. canadiensis*, *C. lupicaris*, *C. neopulicaris*, *C. padusae* and *C. pulicaris*). Three species resemble *C. punctatus*, therefore grouped in the Punctatus group (*C. almeidae*, *C. punctatus* and *C. subpunctatus*). Two species are an intermediate form between the two former species groups (*C. boyi* and *C. yukonensis*).

**Table 1.** Distribution of Pulicaris and Punctatus groups species worldwide. *present in Spain, pulicaris group (dark spot in cubital cel and vthe tips of veins oM1 and M2 dark), punctatus group (dark spot in the cubital cel and the tips of veins M1and M2 pale).

| Species | Group | Distribution |
|---|---|---|
| *C. canadiensis* Wirth and Blanton, 1969 | pulicaris | Nearctic region |
| *C. lupicaris*\* Downes & Kettle, 1952 | pulicaris | Palaearctic region |
| *C. neopulicaris* Wirth, 1955 | pulicaris | Nearctic region |
| *C. padusae* Mirzaeva, 1989 | pulicaris | Palaearctic region |
| *C. pulicaris*\* (Linnaeus), 1758 | pulicaris | Palaearctic region |
| *C.almeidae* Cambournac, 1970 | punctatus | Palaearctic region |
| *C. punctatus*\* (Meigen), 1804 | punctatus | Palaearctic region |
| *C. subpunctatus* Liu & Yu, 1996 | punctatus | Palaearctic region |
| *C. boyi* Nielsen & Kristensen, 2015 | punctatus-pulicaris | Palaearctic region |
| *C. yukonensis* Hoffman, 1925 | punctatus-pulicaris | Nearctic region |

In the present study, a concise analysis of morphological traits and DNA sequencing was performed for specimens collected in Spain. The *Culicoides* analysed were either from the Pulicaris or Punctatus groups. Results obtained led to the description and genetic

characterization of two new species to science within the subgenus *Culicoides*: *C. cryptipulicaris* and *C. quasipulicaris*.

## Methods

### Morphological analyses

*Culicoides* specimens described in the present study were trapped in Spain between years 2004-2009 using CDC black light traps (John W. Hock Company, Gainesville, FL, USA) (Fig. 1).
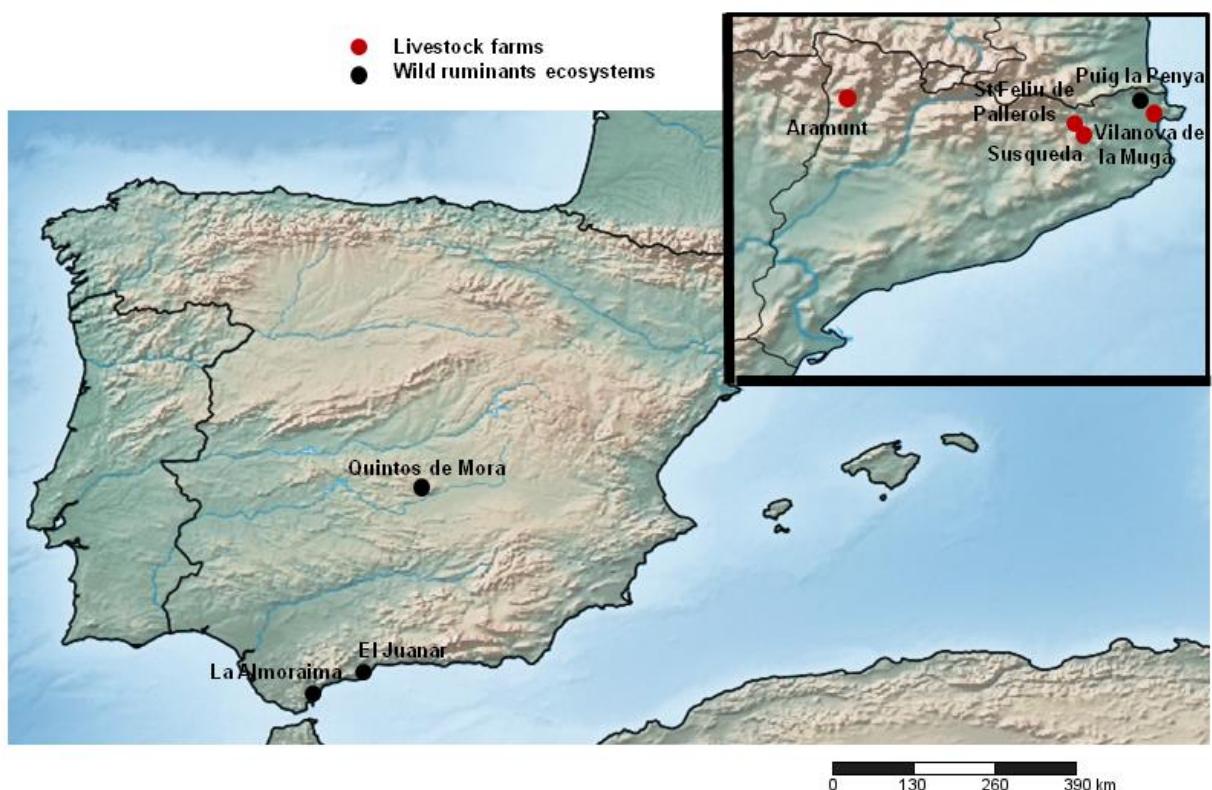


**Figure 1**. Map of Spain with sampling sites.

Midges were morphologically identified under a stereomicroscope (Nikon SMZ) at species-group level according to wing pattern. Individuals with wing pattern resembling *C. pulicaris* or *C. punctatus* were dissected using sterilized ultrafine tweezers. Body parts, except two legs and the anterior part of the abdomen (used for sequencing), were mounted on slides in Tendeiro solution. Metric traits were measured for all slide-mounted individuals using a Nikon Eclipse E200 light microscope (Fig. 2; Tables 2 and 3). When

available, non metric traits were also recorded, primarily male genitalia, for being valuable
diagnostic characters to distinguish species (Fig. 3). Key (male and female) morpholocical
structures of the two new species described where photographed using a Nikon Eclipse 90i
microscope equipped with a Nikon DXM 1200F camera (Tokyo, Japan).  All slides were
deposited in the reference collection of Entomology laboratory of *Centre de Recerca en
Sanitat Animal* (IRTA).
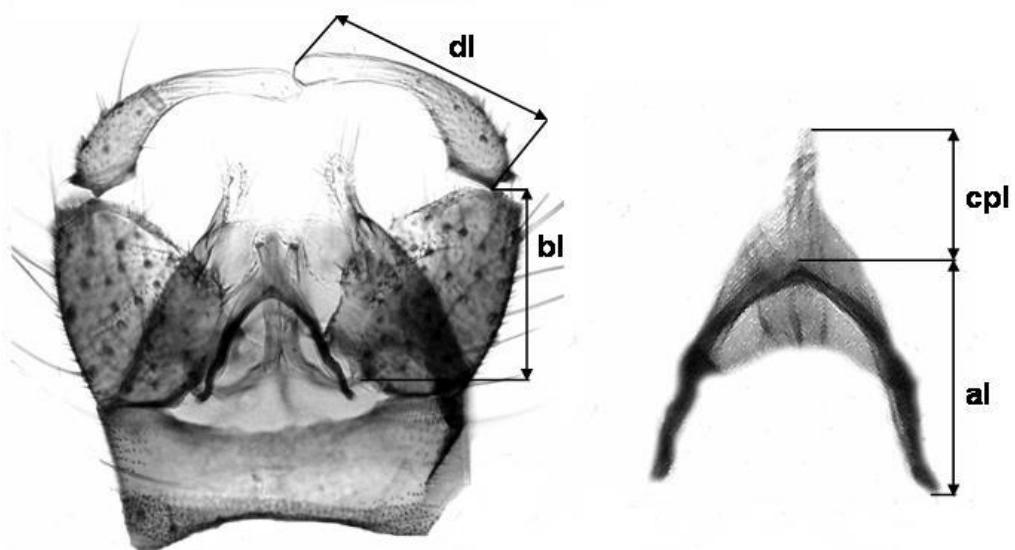


**Figure 2**. *Culicoides* genitalia and aedeagus: **dl**, dististyle length; **bl**, basistyle length, **cpl**, central
proces length; **al**, arms length.

**Table 2**. Metric characters recorded for male *Culicoides*. Characters belong to male antennae, palp, wing and genitalia structures. **AL**, sum of the lengths from the 3rd segment of the flagellum to the 15th; **AR**, ratio of the sum of the lengths of the apical three segments of the flagellum (13-15) to the sum of the basal ten (3-12); **R13/12**, length of the 13th segment of the flagellum to the 12th; **SD**, sensillae coeloconica distribution among the segments of the flagellum; **PL**, sum of the lengths of the five palpal segments; **PR**, ratio of the length of the 3rd palpal segment to the sum of the lengths of the 1st and 2nd; **PR3**, ratio of the length of the 3rd palpal segment to the breadth of the 3rd palpal segment measured at its widest point; **WL**, wing length measured from arculus to apex; **CL**, costa length measured from arculus; **WB**, wing breadth measured at its widest point; **cpl**, aedeagus central proces length (fig.2); **al**, aedeagus arms length (fig.2); **bl**, basistyle length (fig.2); **dl**, dististyle length (fig.2).

|  |  | *C. pulicaris* | *C. lupicaris* | *C. cryptipulicaris* | *C. quasipulicaris* | *C. punctatus* |
|---|---|---|---|---|---|---|
| Antenna | AL (µm) | 980-1055 | 775-985 | 815-940 | 737.50-765 | 752-815 |
|  | AR | 0.61-0.67 | 0.66-0.71 | 0.64-0.67 | 0.59-0.64 | 0.71-0.76 |
|  | R13/12 | 2.20-2.71 | 2.37-2.80 | 2.12-2.50 | 2.22-2.62 | 2.33-2.65 |
|  | SD | 3,13-15 | 3,13-15 | 3,(13)-15 | 3, 13-15 | 3,13-15 |
| Palp | PL (µm) | 230-235 | 167.50-222.50 | 187.50-227.50 | 172.50-190 | 187.50-200 |
|  | PR | 0.81-0.93 | 0.74-0.83 | 0.72-1 | 0.73-0.92 | 0.81-0.96 |
|  | PR3 | 2.36-3.86 | 2.25-3.14 | 2.67-3.67 | 3.17-4.40 | 2.67-3.83 |
| Wing | WL (mm) | 1.58-1.85 | 1.22-1.62 | 1.17-1.47 | 1.02-1.25 | 1.15-1.40 |
|  | CL (mm) | 0.78-1.10 | 0.70-1.00 | 0.62- 0.85 | 0.55-0.65 | 0.62-0.78 |
|  | WB (mm) | 0.55-0.63 | 0.45-0.57 | 0.45-0.52 | 0.40-0.45 | 0.42-0.50 |
| Genitalia | cpl (µm) | 20-37.50 | 27.50-40 | 22.50-27.50 | 30-32.50 | 25-27.50 |
|  | al (µm) | 72.50-80 | 62.50-67.50 | 65-80 | 50 | 60-65 |
|  | bl (µm) | 112.50-145 | 102.50-122.50 | 102.50-132.50 | 95-102.50 | 112.50-122.50 |
|  | dl (µm) | 145-162.50 | 112.50-132.50 | 112.50-140 | 110-115 | 110-135 |
| nº ♂♂ |  | 7 | 7 | 8 | 3 | 5 |

**Table 3.** Metric characters recorded for female *Culicoides*. Characters belong to female eyes, mouthparts, antennae, palp, wing and spermathecae. **F-VL**, fronto-vertex length; **MXT**, maxila teeth number; **MDT**, mandible teeth number; **LL**, labrum length; **AL**, sum of the lengths from the 3rd segment of the flagellum to the 15th; **AR**, ratio of the sum of the lengths of the apical five segments of the flagellum (11-15) to the sum of the basal eight (3-10); **SD**, sensillae coeloconica distribution among the segments of the flagellum; **R11/10**, length of the 11th segment of the flagellum to the 10th; **SD**, sensillae coeloconica distribution among the segments of the flagellum; **PL**, sum of the lengths of the five palpal segments; **PR**, ratio of the length of the 3rd palpal segment to the sum of the lengths of the 1st and 2nd; **PR3**, ratio of the length of the 3rd palpal segment to the breadth of the 3rd palpal segment measured at its widest point; **WL**, wing length measured from arculus to apex; **CL**, costa length measured from arculus; **WB**, wing breadth measured at its widest point; **LS1**, spermathecae 1 length; **LS2**, spermathecae 2 length .

| | | *C. pulicaris* | *C. lupicaris* | *C. cryptipulucaris* | *C. quasipulicaris* | *C. punctatus* |
|---|---|---|---|---|---|---|
| Eyes | F-VL (µm) | 30-47,50 | 15-37.50 | 10-40 | 27.50-30 | 5-32.50 |
| Teeth | MXT | 18-21 | 15-22 | 17-21 | 14 | 18-20 |
| | MDT | 16 | 15-17 | 14-18 | 14 | 14-16 |
| Labrum | LL (µm) | 230-262.50 | 207.50-250 | 200-250 | 167.50-180 | 180-215 |
| Antenna | AL (µm) | 725-820 | 657.50-882.50 | 627.50-845 | 577.50-657.50 | 592.50-772.50 |
| | AR | 0.97-1.10 | 1.03-1.14 | 1-1.15 | 0.97-0.99 | 1.07-1.13 |
| | R11/10 | 1.28-1.50 | 1.40-1.55 | 1.35-1.50 | 1.23-1.28 | 1.27-1.53 |
| | SD | 3, 11-15 | 3, 11-15 | 3, 11-15 | 3, 11-15 | 3, 11-15 |
| Palp | PL (µm) | 262.50-280 | 220-295 | 222.50-282.50 | 192.50-232.50 | 202.50-250 |
| | PR | 0.71-0.87 | 0.67-0.83 | 0.72-0.92 | 0.96-1 | 0.75-0.90 |
| | PR3 | 2.69-3 | 2.36-3.36 | 2.07-3.10 | 1.73-2.35 | 2.64-3 |
| Wing | WL (mm) | 1.42-1.58 | 1.35-1.68 | 1.22-1.88 | 1.12-1.25 | 1.25-1.50 |
| | CL (mm) | 0.87-0.98 | 0.83-1.03 | 0.72-1.10 | 0.60-0.67 | 0.65-0.88 |
| | WB (mm) | 0.63-0.73 | 0.50-0.75 | 0.57-0.85 | 0.52-0.57 | 0.52-0.70 |
| Spermatechae | LS1 (µm) | 67.50-80 | 65-80 | 52.50-80 | 60 | 50-80 |
| | LS2 (µm) | 62.50-67.50 | 52.50-70 | 47.50-72.50 | 55-57.50 | 47.50-67.50 |
| n° ♀♀ | | 6 | 10 | 17 | 2 | 6 |

**Figure 3**. *Culicoides* male genital structures for: whole detail of genitalia (a-e) and aedeagus (f-j). *C. pulicaris* (a, f), *C. lupicaris (b, g)*, *C. cryptipulicaris* (c, h), *C. quasipulicaris* (d, i) and *C. punctatus* (e, j).

A concise bibliographic revision of species resembling either *C. pulicaris* or *C. punctatus* was done on a worldwide basis. Literature used in the study was: [16, 19-21, 27, 29-50].

In accordance with section 8.5 of the ICZN's International Code of Zoological Nomenclature, details of the new species have been registered in ZooBank with the life science identifier (LSID) zoobank.org:pub: XXXXXXX

**DNA extraction, PCR amplification and sequencing**

Total DNA was extracted for dissected *Culicoides* using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Crawley, UK) following manufacturer's protocol in a final elution volume of 100 µl. PCR amplification was performed in a final volume of 50 µl, using 2 mM MgCl2, 1mM dNTPs, 0.2 µM each primer, 1 polymerase unit, and 2 µl of genomic DNA. A fragment of 472bp from COI gene was amplified using forward and reverse primers C1-J-1718 and C1-N-2191 respectively [51]. We also used a new version of the former primers modified by [52] to avoid primer dimerization: C1-J-1718M and C1-N-2191M. Reactions were performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA) with the following amplification program: initial denaturing step at 94ºC for 3 min, followed by 35 cycles at (94 ºC, 30 s; 50 ºC, 30 s; 72 ºC, 30 s) and a final extension step at 72 ºC for 7 min. PCR products were confirmed by electrophoresis and purified using QIAquick gel extraction kit (Qiagen, Crawley, UK) following manufacturer's

instructions. DNA purified products were sequenced on both strands using Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) and analyzed on an ABI PRISM 3730 Automated sequencer (Applied Biosystems).

**Molecular analyses**

The obtained sequences were edited using Bioedit sequence alignment editor software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned with ClustalW Multiple alignment option without manual optimization. Sequences of 472 base pairs covering a fragment of the COI gene were obtained for specimens belonging to the described new species, *C. cryptipulicaris* (former *C. pulicaris* P3 in [25] and *C. quasipulicaris*. Sequences of the former species were aligned with sequences of C. pulicaris, C. boyi, *C. lupicaris*, *C. paradoxalis*, *C. punctatus* and *C. newsteadi* (Table 4). Genetic distance (values of pairwise Tamura-Nei genetic distance, d) and phylogenetic relationships were analyzed. Phylogenetic and molecular evolutionary analyses were conducted using MEGA6 software [53]. Phylogenetic analysis was inferred using Maximum Likelihood (ML) analyses incorporating best fit models of sequence evolution (T92+G) determined using the Akaike Information Criterion with a resampling nodal support of 1000 bootstrap replicates.

**Table 4.** *Culicoides* sample used for phylogenetic analyses.

| Species | NCBI Code | Country |
|---|---|---|
| *C. pulicaris* | GQ338912- GQ338914 | Spain |
| *C. boyi* | JF766347- JF766349, JF766321.1 | Denmark |
| *C. lupicaris* | KF591632, | France, Spain |
| *C. cryptipulicaris n.sp.* | GQ338910, GQ338911 | Spain |
| *C. quasipulicaris n.sp.* | | |
| *C. newsteadi* | GQ338916- GQ338918 | Spain |
| *C. paradoxalis* | KF591673- KF591675 | Portugal |
| *C. punctatus* | GQ338902- GQ338904 | Spain |

## Results

### Description of species

A sample of 71 *Culicoides* was selected according the (morphological) inclusion criteria for Pulicaris and Punctatus groups. Among the individuals analyzed (n = 71), two new species for science were identified and described in the subgenus *Culicoides*: *C. cryptipulicaris* Talavera et al. and *C. quasipulicaris* Talavera et al. Description of species is provided below:


### *Culicoides cryptipulicaris* sp. nov

urn:lsid:zoobank.org:pub:XXXXX

**Male** (8 specimens).

*Head* (Fig. 4a-c, Table 2). Eyes bare and contiguous. Antenna: sensilla coeloconica present on antennal segments 3rd, 13th (one antennae at the most), 14th and 15th, length 815-940µm, Antennal ratio (AR) 0.64-0.67, R13/12 2.12- 2.50. Palp: length 187.50-227.50µm, Palpal Ratio (PR) 0.72-1.00, palpal segment 3th slender with one generally or two sensory pits, PR3 2.67-3.67.

*Thorax* (Fig. 4d, Table 2). Mesonotum brown, generally unmarked*.* Wing pattern like *C. pulicaris*, wing length 1.17-1.47mm , costa length 0.62-0.85mm, breadth 0.45-0.52mm.

*Genitalia* (Fig. 4e-g). Ninth terguite with small lateral process like a thorn. Basistyle with both short ventral and dorsal roots, and some strong bristles on the inner margin. Aedeagus triangular, the arms gently curved and moderately sclerotised but not fusing proximaly. A triangular membrane joins the arms on their proximal third, the central process round-ended. Parameres separated, with some fine bristles at their tip. Ninth sternite with the membrane bare.
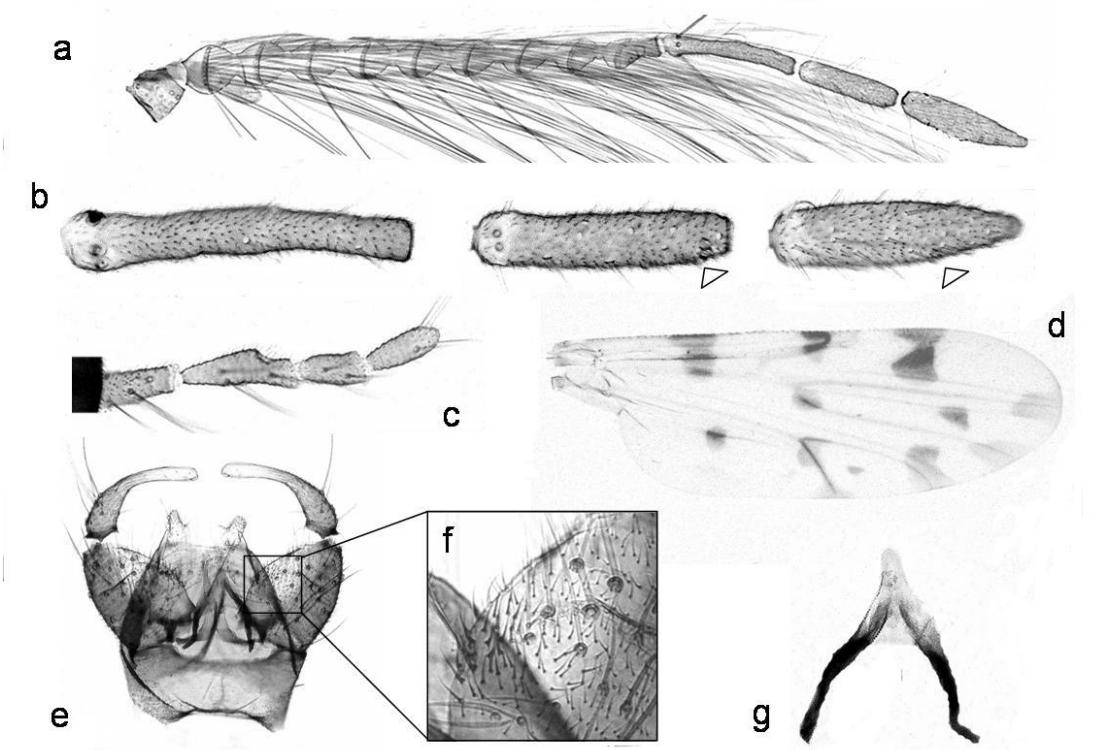
**Figure 4.** Morpholocical structures of *C. cryptipulicaris* male. a: antenna, b: sensilla coeloconica distribution, c: maxillary palp, d: wing, e: genitalia, f: basistyle bristles, g: aedeagus.

**Female** (17 specimens)

***Head*** (Fig. 5a-c, Table 3). Eyes bare and contiguous for a distance of 10- 40μm. Antenna: sensilla coeloconica present on antennal segments 3th, 11th–15th, length 627.50-845μm , Antennal Ratio (AR) 1.00-1.15, R11/10 1.35-1.50. Palp: length 222.50-282.50μm, Palpal Ratio (PR) 0.72-0.92, palpal segment 3th slightly swollen with two or more sensory pits, PR3 2.07-3.10. Labrum: length 200-250μm. Maxilla with 17-21 teeth and mandible with 14-18 teeth.

***Thorax*** (Fig. 5d, Table 3).  Mesonotum brown, generally unmarked*.* Wing with markings simillar to *C. pulicaris*, length 1.22-1.88mm, costa length 0.72-1.10mm, breadth 0.57-0.85mm.

***Abdomen*** (Fig. 5e, Table 3). Spermatechae (S): two, length S1 52.50-80μm and S2 47.50-72.50μm, with short necks, one small rudimentary spermathecae and a ring.
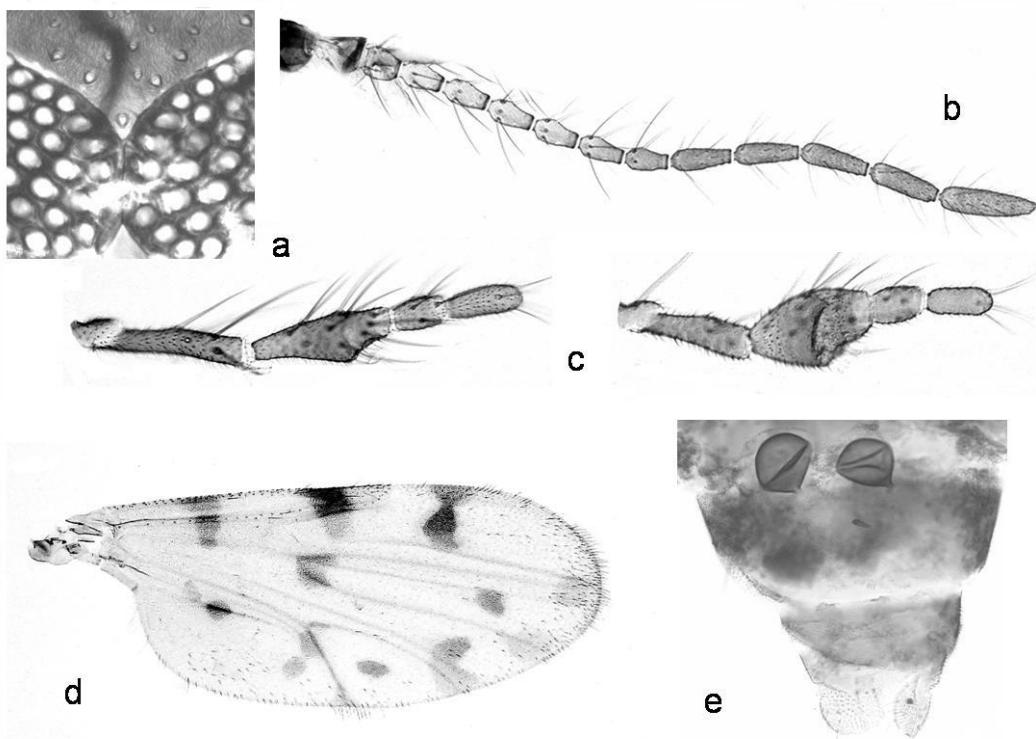
**Figure 5.** Morpholocical structures of *C. cryptipulicaris* female. a: eyes, b: antenna, c: maxillary palp, d: wing, e: spermatheca.

**Type material.**

*Holotype*, ♂ SPAIN: Girona, Terrades, Puig la Penya, ligth-trap, 12-vi-09 (*Muñoz*) (CReSA-1248); *Paratypes*, SPAIN: 1♂ Girona, Sant Feliu de Pallerols, ligth-trap, 09-x-08 (*Muñoz*) (CReSA-1263); 1♂ Girona, Susqueda, ligth-trap, 23-vii-09 (*Pagès*) (CReSA-1266); 2♂ Girona, Susqueda, ligth-trap, 20-viii-09 (*Pagès*) (CReSA-1267,1268); 2♂ Toledo, Los Yébenes, Quintos de Mora, ligth-trap, 01-viii-09 (*Durán*) (CReSA-1255,1256); 1♂ Málaga, Ojén, El Juanar, ligth-trap,13-vii-09 (*Guerrero*) (CReSA-1250); 1♀ Girona, Susqueda, ligth-trap, 22-vii-04 (*Muñoz*) (CReSA-539); 3♀ Girona, Susqueda, ligth-trap, 02-viii-07 (*Pagès*) (CReSA-811,813,830); 1♀ Girona, Vilanova de la Muga, ligth-trap, 09-xii- 04 (*Pagès*) (CReSA-599); 1♀ Girona, Vilanova de la Muga, ligth-trap, 16-xii- 04 (*Pagès*) (CReSA-598); 6♀ Lleida, Aramunt, ligth-trap, 11-x-07 (*Pagès*) (CReSA-803,804,805,808,810,815); 4♀ Málaga, Ojén, El Juanar, ligth-trap, 13-vii-09 (*Guerrero*) (CReSA-1273,1274,1275,1278); 1♀ Toledo, Los Yébenes, Quintos de Mora, ligth-trap, 01-viii-09 (*Durán*) (CReSA- 1279). Holotype and Paratypes deposited at CReSA.

**Remarks on Diagnosis**

*Culicoides cryptipulicaris* is closely related to *C. pulicaris*, the molecular analyses confirming them as sister species. Females of both species do overlap in all recorded characters and measured values (Table 3). This is in accordance with [25], where *C. pulicaris* P3 corresponds to *C. cryptipulicaris*. Then females of both species are morphologically indistinguishable by means of traditional taxonomic traits, and can be considered as true cryptic species. However, *C. cryptipulicaris* males could be identified because they lack sensilla coeloconica in antennal segment 13th either in one or both antennae (Table 2, Fig. 4b). The state of the former trait was confirmed to be constant in all *C. cryptipulicaris* males according to DNA sequences. With regard to males, no diagnostic metric traits could be confidently identified due to the scarce number of specimens available.

**Biology and known distribution**

*C. cryptipulicaris* is widely distributed across Spain being collected over a large range of latitudes (from Northeast to South Spain). It was collected at rural livestock farms (Vilanova de la Muga, Aramunt, Susqueda, Sant Feliu de Pallerols) and natural landscapes with wild ruminants present (El Juanar, Quintos de Mora, Puig la Penya) (Fig. 1). Specimens were collected since late spring (June) to early winter (December).

**Etymology**

This species is named *cryptipulicaris*, resulting from the combination of 'crypti' latin term for cryptic and 'pulicaris', the name of the closest described *Culicoides* species.


*Culicoides quasipulicaris* **sp. nov**

urn:lsid:zoobank.org:pub:XXXXX

**Male** (3 specimens).

*Head* (Fig. 6a-b, Table 2). Eyes bare and contigous. Antenna: sensilla coeloconica present on antennal segments 3rd, 13th, 14th and 15th, length 737.50-765µm, Antennal Ratio (AR) 0.59-0.64, R13/12 2.22-2.62 . Palp: length 172.5-190µm, Palpal Ratio 0.73-0.92, palpal segment 3rd slender with generally one sometimes two sensory pits, PR3 3.17-4.40.

*Thorax* (Fig. 6d, Table 2). Mesonotum brown, generally unmarked. Wing showing intermediate markings between *C. pulicaris* and *C. punctatus* wing pattern; length 1.02-1.25mm, costa length 0.55-0.65mm, breadth 0.40-0.45mm (Table 2).

*Genitalia* (Fig. 6d-f). Ninth terguite with small lateral process like a thorn. Basistyle with both short ventral and dorsal roots, and some strong bristles on the inner margin. Aedeagus triangular, the arms sclerotised (fusing) in contact in their proximal third. Arms gently curved with a crescent-shaped membrane joining them, the central process round-ended. Parameres separated, with some fine bristles at their tip. Ninth sternite with the membrane bare.
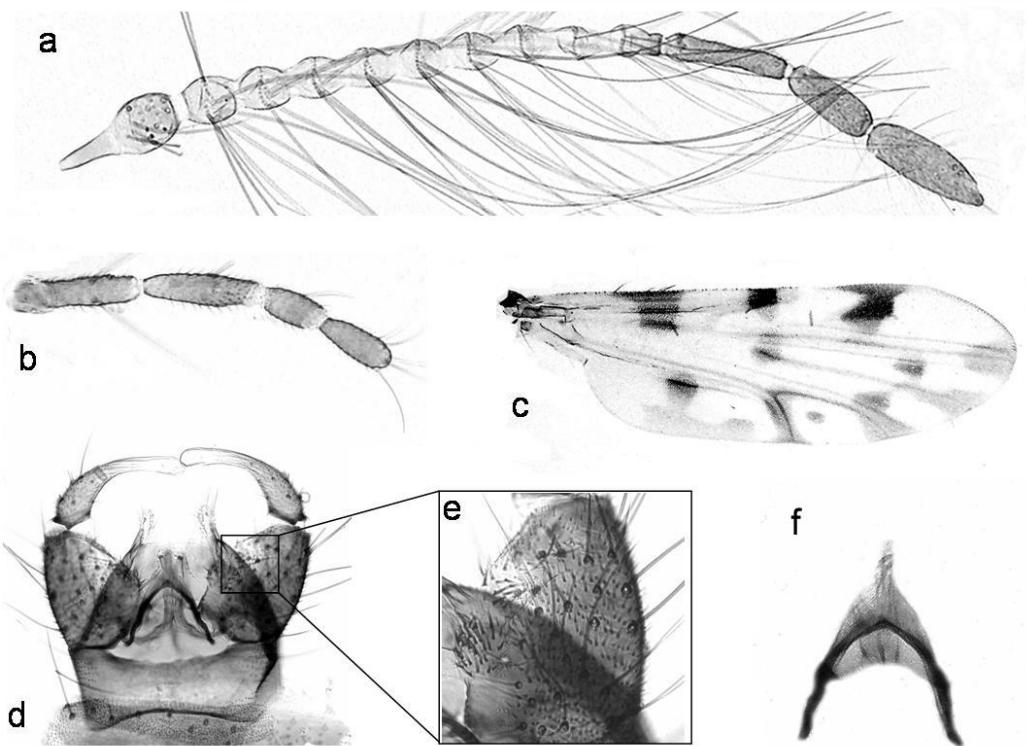


**Figure 6.** Morpholocical structures of *C. quasipulicaris* male. a: antenna, b: maxillary palp, c: wing, d: genitalia, e: basistyle bristles, f: aedeagus.

**Female** (2 specimens)

*Head* (Fig. 7a-c, Table 3). Eyes bare and contigous for a distance of 27.5-30μm. Antenna: sensilla coeloconica present on antennal segments 3rd, 11th -15th, length 577.5-657.5μm, Antennal Ratio (AR) 0.97-0.99, R11/10 1.23-1.28. Palp : length 192.5-232.5μm, Palpal Ratio (PR) 0.96-1, palpal segment 3rd slightly swollen with one or two sensory pits, PR3 1.73-2.35. Labrum: length 167.5-180μm. Maxilla and mandible both with 14 teeth.

**Thorax** (Fig. 7d, Table 3). Mesonotum brown, unmarked*.* Wing showing intermediate markings between *C. pulicaris* and *C. punctatus* wing pattern, length 1.12-1.25mm, costa length 0.60-0.67mm, breadth 0.52-0.57mm.

***Abdomen*** (Fig. 7e, Table 3)***.*** Spermatechae: two, length S1 60µm and S2 55-57.5µm, with short necks, one small rudimentary spermathecae and a ring.
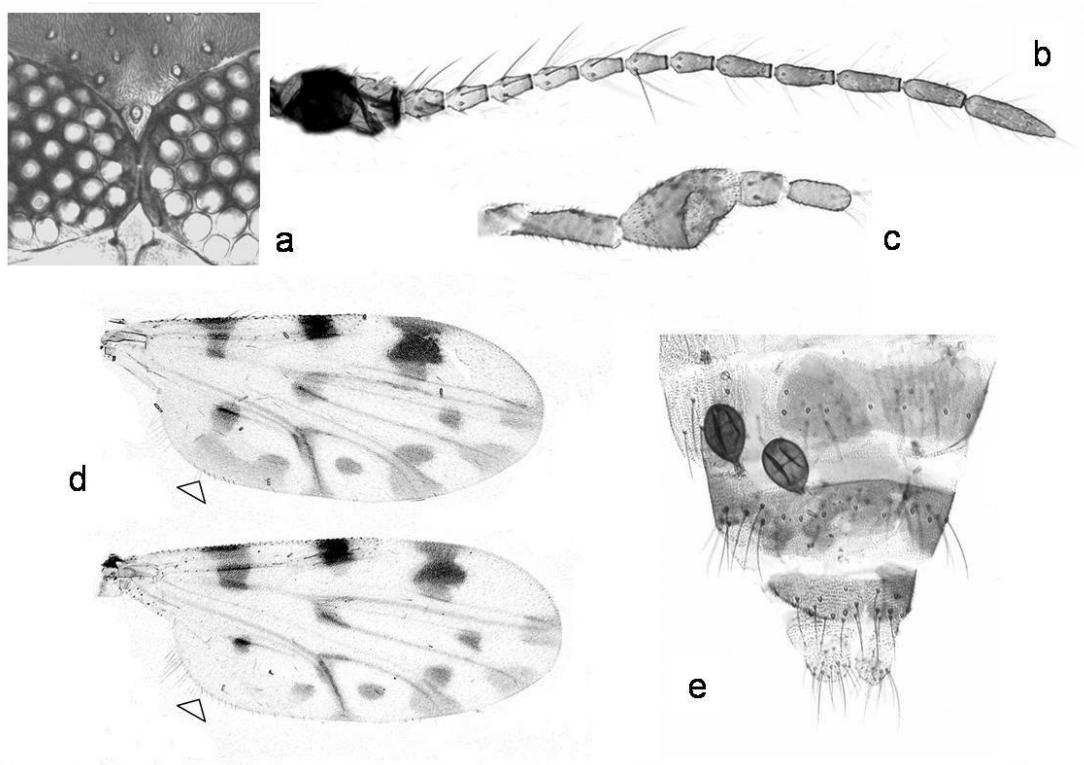


**Figure 7**. Morpholocical structures of *C. quasipulicaris* female. a: eyes, b: antenna, c: maxillary palp, d: wing, e: spermatheca.

**Type material**

*Holotype*, ♀, SPAIN: Toledo, Los Yébenes, Quintos de Mora, ligth-trap, 01-Viii-09 (*Durán*) (CReSA-1282); *Paratypes*, SPAIN: 1♀, Toledo, Los Yébenes, Quintos de Mora, ligth-trap, 01-viii-09 (*Durán*) (CReSA-1280); 1♂, Cádiz, Castellar de la Frontera, La Almoraima, ligth-trap, 14-viii-09 (*Talavera*) (CReSA-1259); 2♂♂, Toledo, Los Yébenes, Quintos de Mora, ligth-trap, 01-viii-09 (*Durán*) (CReSA-1257,1258). Holotype and Paratypes in CReSA.

**Remarks on Diagnosis**

*Culicoides quasipulicaris* is a species morphologically related to *C. pulicaris*. However *C. quasipulicaris* (males and females) could be distinguised from *C. pulicaris* and *C. cryptipulicaris* based on wing pattern. Wing markings represent an intermediate form between *C. pulicaris* (thus *C. cryptipulicaris*) and *C. punctatus* pattern. The r5 spot hourglass-shaped was incomplete as in *C. punctatus*, whereas tips of veins M1 and M2 were dark like in *C. pulicaris*. It is worth to mention that the *C. lupicaris*-typical long dark

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spot in the anal cell running along and in contact with the hind margin was present just in some of the individuals examined, being therefore a variable character (Fig. 8). Additional diagnostic characters to distinguish the species from *C. pulicaris* and *C. punctatus* were present but require microscopic slide preparation. For females, such characters are: Labrum length, Palpal Ratio (PR), Costa length (Table 3). For males are: Antenna length and Aedeagus arms length (Table 2).

Interestingly, individuals matching the described wing pattern have been found in previous studies [30]. Such individuals were identified as *C. pulicaris* males, whereas individuals remained undetermined in [16]. Recently, females with similar wing pattern and without any morphologic distinguishable character have been described as a new species named *Culicoides boyi* [29]. The phylogenetic analyses based on a fragment of the COI gene between *C. quasipulicaris* and *C. boyi* allows to conclude that both entities are valid and different.
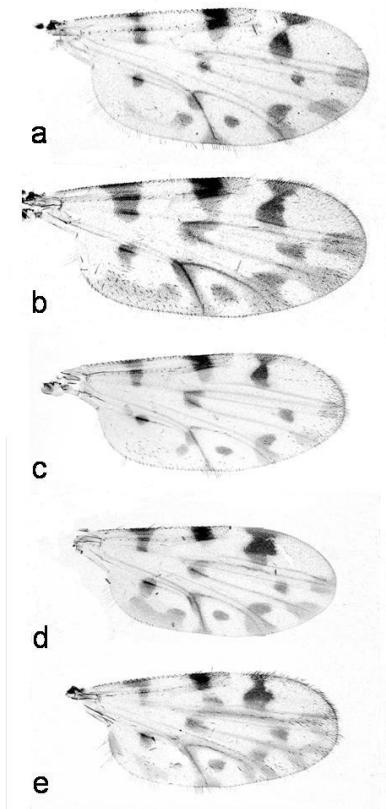


**Figure 8.** Pictures of *Culicoides* wings. a**:** *C. pulicaris*, **b:** *C. lupicaris*, **c:** *C. cryptipulicaris*, **d:** *C. quasipulicaris*, **e:** *C. punctatus*

**Biology and known distribution**

The species has been collected in the Center and South of Spain in natural landscapes with wild ruminants present (La Almoraima, Quintos de Mora) (Fig. 1).

Phenology of the species would remain uncertain due to the scarce number of individuals collected. Nevertheless their summer activity is confirmed, mainly in August.

**Etymology**

The name of this species combines 'quasi' Latin term for almost, or resembling, and 'pulicaris, the name of the morphologically closest described *Culicoides* species.

**COI sequence divergence and phylogenetic analysis**

*Culicoides* sequences were aligned against *Anopheles gambiae* Giles s.s. (DQ792577) to locate translation reading frame, no insertions or deletions were detected. Then, nucleotide sequences were translated into amino acid sequences using the invertebrate mitochondrial code, no stop codons were detected and their structure was in accordance with that suggested by [54]. To validate the proposed descriptions and raise two new species within the subgenus *Culicoides*, distances between candidates representing their most evolutionary closely relative's (species) were checked. Such inter-specific pairwise genetic distances between species (Table 5) ranged from 0.119 (*C. cryptipulicaris* - *C. pulicaris*) to 0.230 (*C. cryptipulicaris* - *C. boyi*). The species *C. quasipulicaris* showed equidistant genetic distance (0.227) for both *C. cryptipulicaris* and *C. punctatus*. Fig. 9 depicts the phylogenetic tree inferred using Maximum Likelihood (ML) analyses with COI sequences. *Anopheles gambiae* Giles s.s. (DQ792577) was used as outgroup and the resulting dendogram showed 8 terminal clades, each one with individuals representing a single species within the *Culicoides* subgenus. A sister association between *C. pulicaris* and *C. cryptipulicaris* is strongly supported although the inclusion of the other two representatives of the Pulicaris complex (*C. lupicaris*, *C. quasipulicaris*) was not supported. *C. punctatus* was apparently (because of low bootstrap support) the closest taxon as expected from previous studies [25].

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**Table 5.** Distance matrix of pairwise genetic distances between Culicoides species. Genetic distances between species are displayed in the bottom half-matrix. Within species genetic distances are displayed in the diagonal (bold).

|  |  | [1] | [2] | [3] | [4] | [5] | [6] | [7] | [8] |
|---|---|---|---|---|---|---|---|---|---|
| [1] | *C. pulicaris* | **0,009** | | | | | | | |
| [2] | *C. boyi* | 0,189 | **0,000** | | | | | | |
| [3] | *C. lupicaris* | 0,166 | 0,153 | **0,002** | | | | | |
| [4] | *C. cryptipulicaris n.sp.* | 0,119 | 0,230 | 0,187 | **0,002** | | | | |
| [5] | *C. quasipulicaris n.sp.* | 0,199 | 0,192 | 0,189 | 0,227 | **0,013** | | | |
| [6] | *C. newsteadi* | 0,200 | 0,213 | 0,183 | 0,200 | 0,251 | **0,005** | | |
| [7] | *C. paradoxalis* | 0,196 | 0,169 | 0,168 | 0,196 | 0,188 | 0,209 | **0,003** | |
| [8] | *C. punctatus* | 0,206 | 0,201 | 0,204 | 0,170 | 0,227 | 0,195 | 0,173 | **0,008** |
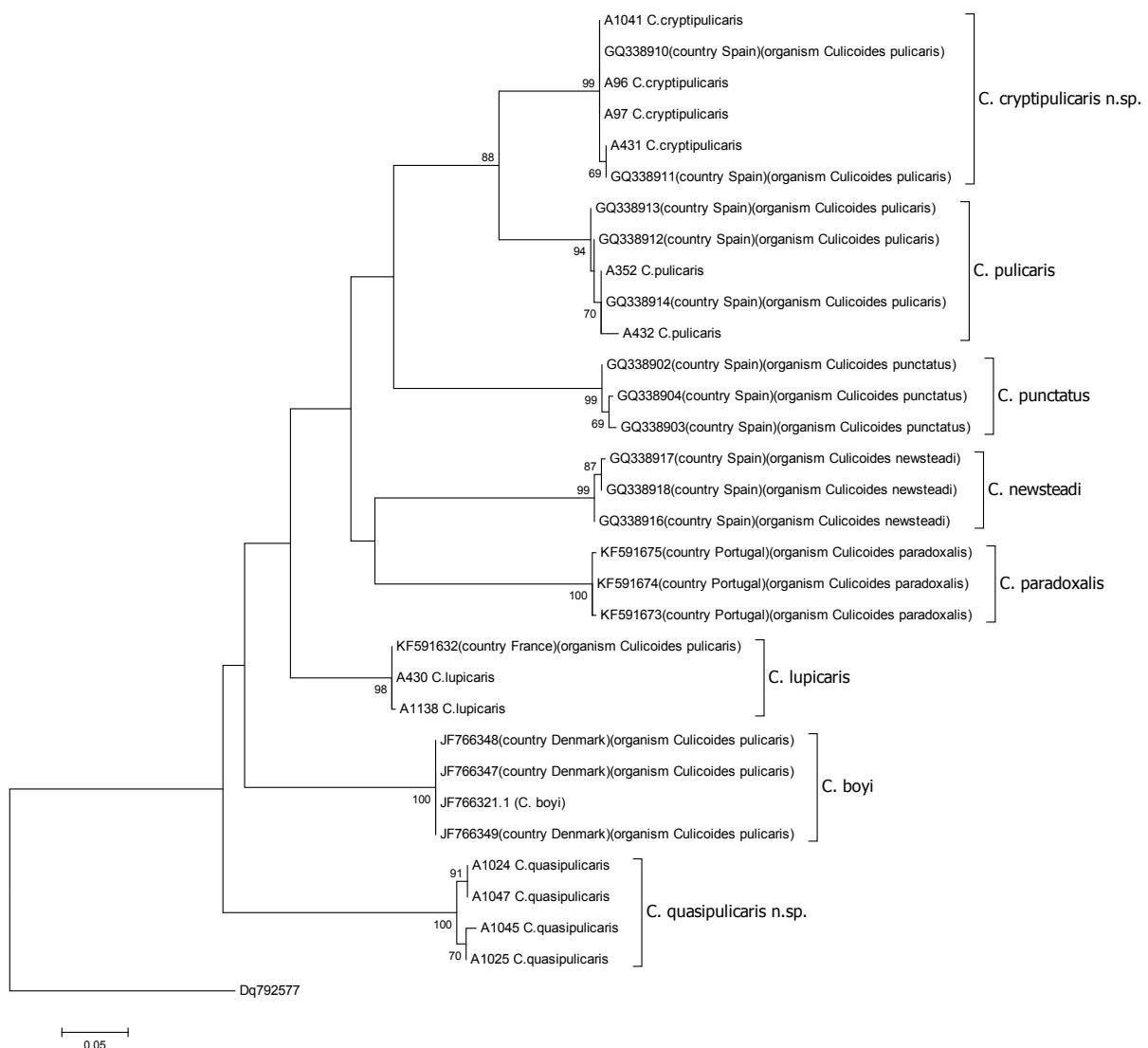


**Figure 9.** Phylogenetic (ML) tree for *Culicoides* inferred from COI sequences.

**Dichothomic Keys**

A species check list of worldwide *Culicoides* resembling individuals within either Pulicaris or Punctatus groups was prepared. The species included were C*. almeidae, C. boyi, C. canadiensis, C. neopulicaris, C. padusae, C. subpunctatus* and *C. yukonensis*. Original decriptions of the former species were concisely analysed and contrasted with spanish individuals before starting *C. cryptipulicaris* and *C. quasipulicaris* descriptions. To evidence important morphological differences that supports the raise of *C. cryptipulicaris* and *C. quasipulicaris* to the status of new species within subgenus *Culicoides*, a brief discussion is provided for each of the *Culicoides* species of the check-list (suplementary material 1, S1).

Based on differences found on certain qualitative (non metric) characters in the present work and the above cited literature, a taxonomical dichothomic key was developed. Sepparate keys for adult male and female individuals is provided (see below). The keys aimed at distinguish species in the Pulicaris and Punctatus groups worlwide. Two species have been excluded, *C. almeidae* (Cambournac, 1970), and *C. yukonensis* (Wirth & Blanton, 1969) because of incomplete information in the description.

**Key of males**

1. Wing with tips of veins M1 and M2 always pale (Fig. 8e) _____**2**

-  Wing with the tips of veins M1 and M2 always dark (Fig. 8a-d) _____**3**

2. Ninth sternite without central process _____***C. puncatus***

-  Ninth sternite with central process _____***C. subpunctatus*** (Liu & Yu,1996)

3. Wing with dark spots non defined and vague_____***C. canadiensis*** (Wirth & Blanton, 1969)

-  Wing with dark spots defined_____**4**

4. Ninth tergite very convex, with apico-lateral process nearly absent _____***C. neopulicaris*** (Wirth & Blanton, 1969)

-  Ninth tergite straight or slightly convex, with apico-lateral process well developed_____**5**

5. Wing with r5 spot hourglass-shaped incomplete (Fig. 8d) _____***C. quasipulicaris***

-  Wing with r5 spot hourglass-shaped complete (Fig. 8 a-c) _____**6**

6. Wing with a long dark spot in the anal cell running along and in contact with the hind margin (Fig. 8b). Aedeagus arms in contact at their proximal part, widening in the mid part (Fig. 3b, g) _____*C. lupicaris*

- Wing lacking a long dark spot in the anal cell running along and in contact with the hind margin (Fig. 8a, c). Aedeagus arms not in contact at their proximal part, no widening (Fig. 3a, c, f, g) _____**7**

7. Sensilla coeloconica present on antennal segments 3$^{th}$, 13$^{th}$-15$^{th}$ _____*C pulicaris*

- Sensilla coeloconica present on antennal segments 3$^{th}$, 14$^{th}$ –15$^{th}$ (one or two antennae (Fig. 4b ) _____*C. cryptipulicaris*


**Key of females**

1. Wing with tips of veins M1 and M2 always pale (Fig. 8e) _____**2**

- Wing with tips of veins M1 and M2 always dark (Fig. 8a-d) _____**3**

2. Genital chitinous plates not tapered _____*C. punctatus*

- Genital chitinous plates tapered_____*C. subpunctatus* (Liu & Yu,1996)

3. Dark spots non defined and diffuse_____*C. canadiensis* (Wirth & Blanton, 1969)

- Dark spots defined_____**4**

4. Sensilla coeloconica present on antennal segments 3$^{th}$, 5$^{th}$ 7$^{th}$,9$^{th}$ -15$^{th}$ _____*C. padusae* (Mirzaeva, 1989)

- Sensilla coeloconica present on antennal segments 3$^{th}$, 11$^{th}$ -15$^{th}$_____**5**

5. Wing with r5 spot hourglass-shaped incomplete. (Fig. 8d)_____ *C. quasipulicaris*, *C. boyi* (Nielsen & Kristensen, 2015)

- Wing with r5 spot hourglass-shaped complete (Fig. 8a-c)_____**6**

6.Wing with a long dark spot in the anal cell running along and in contact with the hind margin (Fig. 8b) _____*C. lupicaris*

- Wing without a long dark spot in the anal cell running along and in contact with the hind margin (Fig. 8a,c) ____*C. pulicaris, C. cryptipulicaris, C. neopulicaris* (Wirth & Blanton, 1969)

**Discussion**

In the present study, morphology and DNA barcodes confirmed the presence of undescribed cryptic species of *Culicoides* in the subgenus *Culicoides*. This subgenus has a long history of taxonomical reorganizations (references above) and is currently composed of 59 species [1, 29, 50]. However, the number of species in the subgenus will rise to 61 for the inclusion of the two newly described species: *C. cryptipulicaris* and *C. quasipulicaris*. Despite such taxonomical reorganizations, species within Pulicaris and Punctatus groups remained always in the subgenus *Culicoides*. Therefore, based on the morphological and phylogenetic data obtained, *C. cryptipulicaris* and *C. quasipulicaris* should be considered as typical members of the subgenus *Culicoides*.

COI gene sequencing has been invaluable to precisely identify and describe *C. cryptipulicaris* and *C. quasipulicaris*. Phylogenetic reconstruction showed well-defined and consistent terminal clades in the dendrogram for each of the eight *Culicoides* species included, with high bootstrap support. Here, the COI gene has proven useful in delimiting species boundaries but not for reconstructing phylogenetic relationships between the species. Consistently low bootstrap values were found for all intermediate clades except the one grouping *C. cryptipulicaris* with *C. pulicaris*. In addition, interspecific genetic distances supported the statuts of *C. cryptipulicaris* and *C. quasipulicaris* as independent species (Table 4). As expected, the lowest evolutionary distances and closest phylogenetic relationships inferred were recorded between *C. cryptipulicaris* and *C. pulicaris*. The estimates of evolutionary divergence over sequence pairs between *C. cryptipulicaris* and *C. pulicaris* was low (d = 0.119). In fact, this value was in accordance with those obtained in previous manuscripts for *Culicoides* cryptic species in the *Avaritia* subgenus [55, 56]. The value was slightly lower than the distance between *C. obsoletus* and *C. scoticus* (d=0.141), where females of these cryptic species still remain morphologically indistinguishable when using classical taxonomic traits [57]. In contrast, such value was higher than the inferred distance for *C. bolitinos* and *C. tuttifruti*, with d = 0.080 (data not shown).

Some of the morphologic metric characters analized revealed interspecific variation for the new described species. These values have been presented elsewhere in the manuscript. However, the authors didn't infer ranges or treshold values for species because of the probable bias associated to the small sample size. In addition, the limited geographic range (Spain) might not be representative of the hypotetic intraspecific range

variation for such species at a global scale. In that sense, it is worth to mention that the species *C. cryptipulicaris* is apparently present in France as well, where it was recently sequenced as *C. pulicaris* (Accesion nº KF591611). Besides metric characters, diagnostic qualitative (non-metric) traits were listed and proved to be useful to distinguish among the species in the Pulicaris group present in Spain (*C. cryptipulicaris*, *C. lupicaris*, *C. pulicaris*, *C. punctatus* and *C. quasipulicaris*). Defining diagnostic qualitative characters for these species is particularly of interest because the Pulicaris group is epidemiologically relevant. The diagnostic traits to distinguish *C. cryptipulicaris* and *C. quasipulicaris* from the other species were sensilla coeloconica distribution, wing pattern (Fig. 8) and male genitalia (Fig. 3).

In Europe, *C. pulicaris* and *C. punctatus* have been pointed as potential BT and SB vectors [4, 12]. These are two of the few *Culicoides* species specifically surveilled during almost the last decade in the framework of the BT entomological surveillance in many EU member states. Such surveillance was mandatory according to decission (EC) nº 1266-2007. Suddenly, we realize that nobody can be sure which species indeed was identifyed as *C. pulicaris* or even *C. punctatus* in Europe. There is clear evidence for an important cryptic species diversity in the subgenus *Culicoides* [25, 28, 29]. Other studies suggest a similar situation even in the Avaritia subgenus [58]. The presence of undetected cryptic species can be a reason when heterogeneous infection rates are found in different geographic areas for a given species. Significant differences in the susceptibility to BTV infection were found for *C. pulicaris* and *C. punctatus* populations in the United Kingdom [8]. The same pattern was described for *C. variipennis* populations in the United States of America [59].

Therefore, future research should provide the necessary information to elucidate wich are the species in the Pulicaris group that certainly have a role in disease transmission.

**Conclusion**

Taxonomy is reinforced when combining traditional morphological approaches with molecular barcoding techniques. Following this joint approach, two new species of *Culicoides* have been described, *C. cryptipulicaris* and *C. quasipulicaris*. Now the question is not wether this represents the tip of the iceberg on *Culicoides* taxonomy, but clearly evidences undescribed species could be more common than expected *a priori*.

The present work is undoubtely of interest for taxonomy, but it is of great interest for animal health. The new species described belong to the subgenus *Culicoides* and are phylogenetically closely related to species that act as potential disease vectors in Europe.

**Additional Files**

**Additional file 1: Supplementary Material 1**

*C. almeidae* (Cambournac, 1970)

Based on description in [37], female wing pattern and mesonotum description are closer to *C. puncatuts* than *C. pulicaris*. Male is unknown. To our criteria, there is not enough information to confirm it is a different species from *C. punctatus*. There is no information on type material and since [37] no further individuals have been identified. None of two new species described in the present study match the description of *C. almeidae*.

*C. boyi* (Nielsen & Kristensen, 2015)

Major female characters do not allow us to distinguish this species from *C. quasipulicaris*. The wing pattern is similar to *C. quasipulicaris*, the dark spot in r5 is broadest above the longitudinal fold above M1 and pale spots at he tip of M1 and M2 are absent [29]. Wing pattern is useful to differentiate the species from the others in Pulicaris-Punctatus groups except *C. quasipulicaris*. The male remains unknown. Fortunately, COI sequences are available from [27]. COI sequence comparison of *C. boyi* with the ones from the present study revealed *C. boyi* is a different species from the individuals analyzed from Spain.

*C. canadiensis* (Wirth & Blanton, 1969)

The species belongs to the Pulicaris complex, a dark spot in the cubital wing cel is present and the tips of the veins M1 and M2 are dark. Spots of the wing are diffused and not defined, more similar to *C. impunctatus* wing pattern than *C. pulicaris* one [36]. Regarding male genitalia, we noted that arms of aedeagus coalesce in the proximal part. Male and female wing spots are useful to distinguish the species from *C. cryptipulicaris* and *C. quasipulicaris*. In addition, the aedeagus arms and fine bristles present at paramers tip separates *C. canadiensis* from *C. cryptipulicaris.*

### *C. neopulicaris* (Wirth, 1955)

The species belongs to the Pulicaris complex, a dark spot in the cubital wing cel is present and the tips of the veins M1 and M2 are dark. Wing spots  are well defined and contrast as in *C. pulicaris*. In females, wing pattern differenciates *C. neopulicaris* from *C. quasipulicaris*, but not from *C. cryptipulicaris* according to available literature [36]. Male genitalia differs from the rest of the Pulicaris complex species,  *C. neopulicaris* ninth tergite is very convex and apicolateral process practically absent, much more similar to species resembling *C. cockerellii* [36]. Male genitalia is realiable to distinguish *C. neopulicaris* from *C. cryptipulicaris* and *C. quasipulicaris*.

### *C. padusae* (Mirzaeva, 1989)

The species belongs to the Pulicaris complex, a dark spot in the cubital wing cel is present and tips of the veins M1 and M2 are dark. Male still remains unknown. In females, distribution of sensilla coeloconica (SD) is  3th, 5th, 7th, 9th -15 [th] [42], enought to distinguish *C. padusae* from *C. cryptipulicaris* and *C. quasipulicaris* (Table 3).

### *C. subpunctatus* (Liu & Yu,1996)

The species belongs to the Punctatus complex, a dark spot is present in the cubital wing cel and the tips of the veins M1 and M2 are pale. The wing pattern is useful to differentiate both *C. cryptipulicaris* and *C. quasipulicaris* from *C. subpunctatus*. We also found differences on sub-genital chitinous plates of the female genitalia and on aedeagus and ninth sternite of male [47] that allow us to differentiate *C. punctatus*.

### *C. yukonensis* (Hoffman, 1925)

Different wing patterns have been attributted to *C. yukonensis*. Most of the individuals had the wing pattern typical of the Punctatus complex, however some individuals had wing pattern resembling more that of the Pulicaris complex. Wing patterns attributed to *C. yukonensis* are different than that of *C. quasipulicaris*. Wing pattern does not confidently distinguish *C. yukonensis* from *C. cryptipulicaris*. In that case, the  female AR and  the proximal fusion of aedeagus arms in male genitalia allows us to differentiate *C. yukonensis* from *C. cryptipulicaris* [36].

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

ST and NP conceived the study. ST, FMM and MV identified *Culicoides* specimens. ST, FMM and NP analysed the data, interpreted the results and wrote the first draft of the paper. All authors read and approved the final manuscript.

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**Capítol III**

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# Culicoides Species Communities Associated with Wild Ruminant Ecosystems in Spain: Tracking the Way to Determine Potential Bridge Vectors for Arboviruses

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## Abstract

The genus Culicoides Latreille 1809 is a well-known vector for protozoa, filarial worms and, above all, numerous viruses. The Bluetongue virus (BTV) and the recently emerged Schmallenberg virus (SBV) are responsible for important infectious, non-contagious, insect-borne viral diseases found in domestic ruminants and transmitted by Culicoides spp. Both of these diseases have been detected in wild ruminants, but their role as reservoirs during the vector-free season still remains relatively unknown. In fact, we tend to ignore the possibility of wild ruminants acting as a source of disease (BTV, SBV) and permitting its reintroduction to domestic ruminants during the following vector season. In this context, a knowledge of the composition of the Culicoides species communities that inhabit areas where there are wild ruminants is of major importance as the presence of a vector species is a prerequisite for disease transmission. In this study, samplings were conducted in areas inhabited by different wild ruminant species; samples were taken in both 2009 and 2010, on a monthly basis, during the peak season for midge activity (in summer and autumn). A total of 102,693 specimens of 40 different species of the genus Culicoides were trapped; these included major BTV and SBV vector species. The most abundant vector species were C. imicola and species of the Obsoletus group, which represented 15% and 11% of total numbers of specimens, respectively. At the local scale, the presence of major BTV and SBV vector species in areas with wild ruminants coincided with that of the nearest sentinel farms included in the Spanish Bluetongue Entomological Surveillance Programme, although their relative abundance varied. The data suggest that such species do not exhibit strong host specificity towards either domestic or wild ruminants and that they could consequently play

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a prominent role as bridge vectors for different pathogens between both types of ruminants. This finding would support the hypothesis that wild ruminants could act as reservoirs for such pathogens, and subsequently be involved in the reintroduction of disease to livestock on neighbouring farms.

## Introduction

Around 1,400 species of biting midges of the genus *Culicoides* have been described in the world [1]; some of these are well known transmitters of protozoa, filarial worms and viruses that affect humans and domestic and/or wild animals [2]. One of the most important of these pathogens is Bluetongue virus (BTV), which is a double stranded RNA virus of the genus *Orbivirus* that produce an infectious, non-contagious disease that affects domestic and wild ruminants [3]. At the global scale, BTV is one of the most economically important diseases transmitted by *Culicoides* in terms of the disruption of both international and domestic trade [4]. Over the last decade, BT has re-emerged in the Mediterranean countries. The spread of this disease was initially associated with the introduction and establishment of the main vector for BTV outbreaks in Africa and Southern Europe, the Afro-Asiatic species *Culicoides imicola* Kieffer, 1913. Once this disease had become established in Southern Europe, BTV-8 unexpectedly appeared in Western and Central Europe in August 2006, where *C. imicola* was absent, and where endemic species of *Culicoides* such as *C. obsoletus* and *C. scoticus* [5], [6, 7, 8], *C. dewulfi* [9], *C. chiopterus* [10] and *C. pulicaris* [11] were pointed to as potential vectors for the disease. *Culicoides* have recently been identified as potential carriers of Schmallenberg virus (SBV), based on both field [12–15] and laboratory [16] studies. The virus produces a disease that affects ruminants and which was first detected in Germany and the Netherlands in the summer and autumn of 2011 [17]. Since then, it has spread throughout almost the whole of Europe and its presence was confirmed in Spain (in March 2012) when it affected sheep and goats in the south of the country [18]. To date, eight species of *Culicoides* have been described as vectors for SBV in Europe: *C. obsoletus*, *C. scoticus*, *C. dewulfi*, *C. chiopterus* [12, 13, 14], *C. punctatus* [15], *C. pulicaris*, *C. nubeculosus* and *C. imicola* [16]; all of these are considered vectors of BTV except *C. punctatus* and *C. nubeculosus*.

Seven different species of wild ruminants are present in Spain; the red deer (*Cervus elaphus* Linnaeus, 1758), which is the most abundant species; the fallow deer (*Damma damma* Linnaeus, 1758); the roe deer (*Capreolus capreolus* Linnaeus, 1758); the mouflon (*Ovis aries musimon* Pallas, 1762); the Spanish ibex (*Capra pyrenaica hispanica* Schinz, 1838); the Pyrenean chamois (*Rupicapra pyrenaica* Bonaparte, 1845); and the aoudad or Barbary sheep (*Ammotragus lervia* Pallas, 1777) [19]. The infection of wild ruminants by BTV and SBV has been previously reported and specific antibodies to BTV have been detected in all of the previously listed species in Spain [20–24], and to SBV in several wild ruminant species: red deer, roe deer, fallow deer, European bison, elk, chamois and Pyrenean chamois in other parts of Europe [25–29]. The role played by wild ruminants in relation to the maintenance of disease and its dissemination to domestic ruminants has so far received little attention, although recent studies suggest their involvement in the disemination of BTV and its persistence in Spain [23, 24]. The detection and control of Bluetongue and Schmallenberg in wild ruminants is difficult, particularly as most species are asymptomatic to BTV [30] and SBV [27, 28]; controlling *Culicoides*-borne pathogens that come from wild populations is therefore extremely difficult.

The characteritzation of *Culicoides* midge communities in areas in which wild ruminants are present is important for understanding the role that wild ruminants could play in the

dynamics of BTV and SBV. To our knowledge, the composition of *Culicoides* communities in these areas has so far been poorly studied in Europe and deserves greater attention. The main objective of the present study was therefore to characterize *Culicoides* midge communities in forest environments where wild ruminants were present and abundant and to compare such communities with those found close to livestock. To achieve this main goal, the following specific objectives were established: i) to determine the relative abundance of *Culicoides* species within wild ruminant areas, ii) to reveal whether the main vector species present on livestock farms are also present in wild ruminant areas, and whether they could therefore act as bridge vectors between the two types of ruminants, and finally iii) to determine whether some mammalophilic *Culicoides* species (or ones without known host preferences) are absent from livestock farms in areas also inhabited by wild ruminants.

## Materials and Methods

### Sampling

The *Culicoides* specimens identified in the study were trapped in 2009 and 2010, during the main *Culicoides* activity season (from July to November). They were captured on seven Spanish private areas characterized by their distinctive bioclimatic features and wild ruminant communities (Table 1). Data relating to bioclimatic variables and altitude were obtained from the climatic atlas of the Iberian Peninsula [31]; landscape variables were obtained from the Global Environment Monitoring database [32], and the distribution of the different ruminants within Spain was obtained from an atlas of land mammals in Spain [19]. Permanent single trapping sites were established near water sources in each area; these were usually located more than 1 km from the closest livestock farm. Food and water were provided to wild ruminants on a regular basis at Puig la Penya and El Juanar. Three CDC black light traps (John W. Hock Company, Gainesville, FL, USA) were placed at each sampling site and used from dusk to dawn on three consecutive nights, once per month. The CDC traps were employed to ensure results that would be comparable with data from the Spanish Bluetongue National Surveillance Programme (which also used CDC black light traps). Trapped insects were collected in containers containing soapy water and were then stored in 70% ethanol for morphological identification. Access to private land was granted by the respective landowners. Fieldwork did not involve any endangered or protected species.

In order to compare the composition of the *Culicoides* vector species between areas occupied by domestic and wild ruminants, contemporary data were obtained from the Spanish Bluetongue National Surveillance Programme relating to seven livestock farms (Table 2). These were the farms located closest to the seven study sites with wild ruminants (which were less than 60 km apart). Although the data from the Spanish Bluetongue National Surveillance Programme only included data for known BT vector species, data for all the trapped *Culicoides* species were also available for farms at Vilanova de la Muga and Aramunt (which were included in communitiy analyses).

### Morphological and molecular identification

*Culicoides* midges were first identified, under a stereomicroscope (Nikon SMZ), at the species or species-group level, according to their wing pattern morphology [33] (S1 Table). In addition, females were separated by the gonodotrophic status following the categorization performed by Dyce [34]. In order to perform community analyses at wild ruminant sites, an accurate morphological identification was later performed for all the species cited in the manuscript on dissected individuals slide-mounted in Canada balsam (for at least one individual of each sex). The slides were examined with a Nikon Eclipse E200 light microscope using the main

*Culicoides* Species Associated with Wild Ruminants

Table 1. Data summary of ecological variables and characterization of the sampling sites [19, 32].

| Sampling site | Code | Geographical variables | | | Bioclimatic variables | | | Environment near the sampling site | | Ruminant | Ruminants in sampling place | *Culicoides* | Landscape |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Latitude | Longitude | A(m) | HT (°C) | LT (°C) | AP (mm) | Domestic ruminants (distance in km) | Water (distance in meters) | (wild—domestic) | (more abundant—less abundant) | % total (both sexes) | |
| Proaza | 1W | 43.203361 | -6.055999 | 349 | 25 | 0.0 | 1,000 | Farm (4,5) | wet soil, not water on surface | wild | red deer | 5.9 | Shrub Cover, closed-open, deciduous |
| R.N.C. Boumort | 2W | 42.201691 | 1.099684 | 1,276 | 25 | -7.5 | 900 | Free domestic livestock (1) | Pond (<5) | wild | red deer, fallow deer, roe deer, chamois | 0.1 | Tree Cover, broadleaved, deciduous, closed |
| Puig la Penya | 3W | 42.307891 | 2.803300 | 228 | 28 | 2.5 | 1,000 | Farm (2) | Pond (25) | wild | red deer, fallow deer, mouflon | 4.4 | Tree Cover, needle-leaved, evergreen |
| Quintos de Mora | 4W | 39.383333 | -4.100000 | 718 | 35 | 0.0 | 482 | Farm (10) | Seasonal stream (<5) | wild | red deer, fallow deer, roe deer | 27.6 | Tree Cover, needle-leaved, evergreen |
| La Morera | 5W | 38.911372 | -4.260120 | 707 | 35 | 2.5 | 500 | Farm (4) | Pond (5) | wild | red deer, mouflon, aoudad | 6.5 | Shrub Cover, closed-open, evergreen |
| El Juanar | 6W | 36.569647 | -4.890657 | 870 | 28 | 2.5–5.0 | 850 | Farm (5) | Cement trough (<5) | wild | Spanish ibex | 0.9 | Tree Cover, needle-leaved, evergreen |
| La Almoraima | 7W | 36.289592 | -5.431023 | 45 | 31 | 7.5 | 955 | Farm (4) | wet soil, not water on surface | wild | red deer, fallow deer, roe deer, mouflon | 54.6 | Cultivated and managed areas |
| Tineo | 1D | 43.194797 | -6.251201 | 673 | 25 | 0.0 | 1,000 | - | - | domestic | cow | - | Cultivated and managed areas |
| Aramunt | 2D | 42.206277 | 0.987546 | 559 | 23 | -2.5 | 700 | - | - | domestic | sheep | - | Cultivated and managed areas |
| Vilanova de la Muga | 3D | 42.303944 | 3.031915 | 19 | 25 | 2.5 | 800 | - | - | domestic | sheep | - | Cultivated and managed areas |
| Piedrabuena | 4D | 39.147973 | -4.101201 | 584 | 35 | 0.0 | 400 | - | - | domestic | cow | - | Cultivated and managed areas |
| Navacerrada | 5D | 38.453675 | -4.260202 | 614 | 37.5 | 0.0 | 500 | - | - | domestic | cow, sheep, goat | - | Cultivated and managed areas |
| Mijas | 6D | 36.311594 | -4.421226 | 428 | 32.5 | 5.0 | 700 | - | - | domestic | sheep, goat | - | Cultivated and managed areas |
| Castellar de la Frontera | 7D | 36.191199 | -5.270036 | 47 | 32.5 | 5.0–7.5 | 1,000 | - | - | domestic | sheep, goat | - | Cultivated and managed areas |

A, altitude; AP, annual precipitation; LT, mean low temperature of the coldest month; HT, mean high temperature of the warmest month [31].

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**Table 2. Vector species or species group abundance (n° midge/night/trap) at wild and domestic ruminants sampling sites.**

| Sampling Site | Site | Ruminant site | *C. imicola* | Obsoletus group | Pulicaris group | N (n°midges/night/trap) | % N |
|---|---|---|---|---|---|---|---|
| Tineo | 1D | Domestic | 0.00 | 27.95 | 1.70 | 29.65 | 0.29 |
| Aramunt | 2D | Domestic | 0.00 | 1,081.15 | 71.80 | 1,152.95 | 11.42 |
| Vilanova de la Muga | 3D | Domestic | 0.00 | 15.70 | 1.15 | 16.85 | 0.17 |
| Piedrabuena | 4D | Domestic | 155.00 | 2.00 | 0.00 | 157.00 | 1.56 |
| Navacerrada | 5D | Domestic | 258.75 | 0.60 | 0.00 | 259.35 | 2.57 |
| Mijas | 6D | Domestic | 418.17 | 2.75 | 0.00 | 420.92 | 4.17 |
| Castellar de la Frontera | 7D | Domestic | 52.32 | 0.00 | 0.00 | 52.32 | 0.52 |
| Total domestic ruminant sites | | | 884.24 | 1,130.15 | 74.65 | 2,089.04 | 20.70 |
| Proaza | 1W | Wild | 0.00 | 1,186.59 | 55.60 | 1,242.19 | 12.31 |
| R.N.C. Boumort | 2W | Wild | 0.00 | 7.31 | 5.97 | 13.28 | 0.13 |
| Puig la Penya | 3W | Wild | 0.00 | 546.95 | 83.63 | 630.58 | 6.25 |
| Quintos de Mora | 4W | Wild | 14.31 | 212.95 | 123.27 | 350.53 | 3.47 |
| La Morera | 5W | Wild | 41.62 | 0.00 | 5.97 | 47.59 | 0.47 |
| El Juanar | 6W | Wild | 35.31 | 133.95 | 109.94 | 279.20 | 2.77 |
| La Almoraima | 7W | Wild | 5,196.61 | 133.28 | 110.29 | 5,440.18 | 53.90 |
| Total wild ruminant sites | | | 5,287.85 | 2,221.03 | 494.67 | 8,003.55 | 79.30 |
| | | | | | | 10,092.59 | 100.00 |

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taxonomic keys for Palearctic *Culicoides* [35–39]. It is difficult to separate *C. obsoletus* and *C. scoticus* females using traditional morphological techniques [40]. In order to confirm the presence of Obsoletus group females, precise identification of 73 females of the Obsoletus group was performed by means of PCR according to the procedures described in [41 and 42].

*Culicoides* species were classified into ornithophilic and mammalophilic according to their feeding habits, based on morphological analysis of main sensory structures such as antennae and palps, ([43] and references therein). Species with sensilla coeloconica (SC) on 8 or more antennal flagellomers, SC on antennal flagellomers 4–10 and 1 large maxillary palp sensory pit, were categorised as ornitophilic. Those with SC on 6 or fewer antennal flagellomers, without SC on antennal flagellomers 4–10 and 1 or more smaller maxillary palp sensory pits were categorised as mammalophilic. Species that did not fit into either of these two categories were categorised as indefinite or unknown.

The different specimens that were deposited, at the CReSA collection were cited using the following abbreviation: INIA-CReSA.

## Statistical analyses

For the *Culicoides* community analyses, a presence/absence dataset of all the vector and non-vector species was established, at a species level, for the seven wild ruminant sites and the two domestic ruminant sites (Aramunt and Vilanova de la Muga). The *Culicoides* species richness (number of species per site) was calculated. Similarities between different *Culicoides* communities were determined using the Bray-Curtis (BC) similarity index [44]. Multivariate non-metric multidimensional scaling (nMDS) was used to asses the relationships between the different *Culicoides* communities at all the different sites. MDS allows visualizing the degree of similariy between the samples in a data matrix by displaying the information contained in a distance/similarity matrix. Data were analyzed using a one-way analysis of similariy (ANOSIM) to test for differences between the presence of domestic or wild ruminant and between neighbouring landscapes. This procedure generates an *R* statistic that quantifies the degree of discrimination

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between sites and a *p* value that indicates the significance of the differences observed. The R statistic ranges from 0 to 1 and is approximately zero if the null hypothesis is true: when the similarities within sites tend on average to be the same as those between different sites [45]. The projection of vectors in the nMDS ordination finds the directions in the ordination space towards which the environmental vectors change most rapidly and to which they have maximal correlations with the ordination configuration. Then, vectors (arrows) in the nMDS plot represent explanatory environmental variables (bioclimatic variables and altitude, see Table 1) and are proportional in length to their importance. Similarity profile analysis (SIMPROF) was also carried out to statistically detect structuring in *Culicoides* communities. SIMPROF examines null hypothesis by testing whether the similarities observed in the data are larger or smaller than those that could be expected due to chance. A two-way cluster analysis was performed factoring in both sampling sites and species based on BC similarity index of presence/absence data. Two-way cluster analysis independently groups sample sites and species and combines them in a single diagram to allow the observation of associations between different groups of sample units and species.

The abundance data available for relevant vector species or species groups for the 14 different localities were used to test for differences between wild and domestic ruminant sites. For comparative analyses, the data were transformed into n° midges/trap/night because the trapping effort used in the current work was different from the one used by the Spanish Bluetongue National Surveillance Programme. Prior to analysis, the data matrix containing the abundance of vector species per site was square root transformed to reduce the importance of extreme values [44]. Similarities between sites were determined using the BC similariy index and visualized by nMDS. ANOSIM was carried out to test whether the composition of the *Culicoides* community significantly differed according to the type of ruminant species (domestic or wild) considered.

All the multivariate analyses were performed using the Primer 7 software package [46].

## Results

A total of 102,693 specimens of the genus *Culicoides* were trapped during the study period (S1 Table). Of them, 20,970 (20%) were males and 81,723 (80%) were females, with 79.75% being parous and 0.25% blood engorged females [34]. The specimens were assigned to one of 40 different species (Table 3) without any new species being cited for the Iberian Peninsula with respect to the latest taxonomic catalog published by Alarcón-Elbal and Lucientes [47].

Analyzing the richness of species in each of the areas studied, up to 28 species were detected in La Almoraima and at least of 11 species in R.N.C. Boumort and Proaza (Fig 1, Table 3). The mean number of species per site was greater at wild (18.3) than at domestic (13) ruminant sites. Fig 2 shows the nMDS plot for species composition and, according to the SIMPROF tests ($p < 0.05$), several groups can be separated. Three groups were detected at similarity levels of 60%: Vilanova de la Muga and Aramunt were grouped together, R.N.C. Boumort and Puig la Penya formed another group, and Quintos de Mora, La Morera and La Almoraima were also considered to have similar *Culicoides* communities. At this similarity level, El Juanar was judged to constitute a separate group of its own. Proaza was very different the other groups, with less than 40% similarity. The *Culicoides* communities associated with domestic and wild ruminant sites were similar (ANOSIM, global $R = 0.175$, $p = 0.250$), indicating that the presence of one ruminant type ore another did not affect the species composition (presence/absence data). The ANOSIM results also showed that landscape did not have a significant influence on the observed variations in species composition (global $R = 0.233$, $p = 0.207$). The nMDS (Fig 2) revealed annual precipitation to be the factor that most explained the

*Culicoides* Species Associated with Wild Ruminants

Table 3. Distribution, morphological features and host-feeding preferences of all the identified species of *Culicoides*.

| Species | Distribution found | | | | | | | | | Antenna (Sensilla coeloconica) | | Maxillary palp (Sensory pit) | | Host preference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1W | 2W | 3W | 4W | 5W | 6W | 7W | 2D | 3D | AF with SC | presence/absence AF 4–10 | number | size | |
| *Culicoides alazanicus* Dzhafarov, 1961 | • | | • | | • | | | | • | 13 | presence | 1 | large | birds |
| *Culicoides (Oecacta) brunnicans* Edwards, 1939 | | | | • | | | | | | 9 | presence | 2 | small | indefinite |
| *Culicoides cataneii* Clastrier, 1957 | | • | • | • | • | • | • | | | 12–13 | presence | 1 | large | birds |
| *Culicoides (Beltranmyia) circumscriptus* Kieffer, 1918 | | • | • | • | • | • | • | • | • | 12 | presence | 1 | large | birds |
| *Culicoides coluzzii* Callot, Kremer & Bailly–Choumara, 1970 | • | | | • | • | | • | | | 8 | presence | 1 | large | birds |
| *Culicoides derisor* Callot & Kremer, 1965 | | | • | | | | | | | 6 | absence | 1 | small | mammals |
| *Culicoides (Avaritia) dewulfi* Goetghebuer, 1936 | • | | | | | | | | | 6 | absence | 1 | small | mammals |
| *Culicoides (Culicoides) fagineus* Edwards, 1939 | • | | | • | • | • | | • | • | 6 | absence | various | small | mammals |
| *Culicoides festivipennis* Kieffer, 1914 | • | • | • | • | • | | • | | | 13 | presence | 1 | large | birds |
| *Culicoides (Culicoides) flavipulicaris* Dzhafarov, 1964 | | | | | | | • | | • | 6 | absence | various | small | mammals |
| *Culicoides furcillatus* Callot, Kremer & Paradis, 1962 | • | | | | | | | | | 6 | absence | 1 | small | mammals |
| *Culicoides gejgelensis* Dzhafarov, 1964 | | • | • | • | | • | • | | | 12–13 | presence | 1 | large | birds |
| *Culicoides griseidorsum* Kieffer, 1918 | | | | | | | | | • | 12 | presence | 1–2 | large | birds |
| *Culicoides haranti* Rioux, Descous & Pech, 1959 | | | • | • | • | | | | | 11–13 | presence | 1 | large | birds |
| *Culicoides heteroclitus* Kremer & Callot, 1965 | | | | • | • | | • | | • | 12–13 | presence | 1 | large | birds |
| *Culicoides (Avaritia) imicola* Kieffer, 1913 | | | | • | • | • | • | | | 6 | absence | 1 | small | mammals |
| *Culicoides (Culicoides) impunctatus* Goetghebuer, 1920 | • | | | | | | | • | | 5–6 | absence | various | small | mammals |
| *Culicoides jumineri* Callot & Kremer, 1969 | | | | • | | | | | | 9 | presence | 1 | large | birds |
| *Culicoides kibunensis* Tokunaga, 1937 | | • | | | | | | | | 13 | presence | 1 | large | birds |
| *Culicoides kurensis* Dzhafarov, 1960 | | | • | | • | | • | | | 9 | presence | 1 | large | birds |
| *Culicoides lailae* Khalaf, 1961 | | | | | | | | • | | 12 | presence | 1 | large | birds |
| *Culicoides lupicaris* Downes & Kettle, 1952 | • | | • | | | | | | | 6 | absence | various | small | mammals |
| *Culicoides marcleti* Callot, Kremer & Basset, 1968 | | | | • | | | • | | | 8 | presence | 1 | large | birds |
| *Culicoides maritimus* Kieffer, 1924 | | | • | | | | • | | | 13 | presence | 1 | large | birds |
| *Culicoides (Culicoides) newsteadi* Austen, 1921 | | | • | • | • | | • | • | • | 6 | absence | various | small | mammals |
| *Culicoides (Avaritia) obsoletus* (Meigen, 1818) | • | • | • | • | | • | • | • | • | 6 | absence | 1 | small | mammals |

(Continued)

*Culicoides* Species Associated with Wild Ruminants

**Table 3.** (*Continued*)

| Species | Distribution found | | | | | | | | | Antenna (Sensilla coeloconica) | | Maxillary palp (Sensory pit) | | Host preference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1W | 2W | 3W | 4W | 5W | 6W | 7W | 2D | 3D | AF with SC | presence/ absence AF 4–10 | number | size | |
| *Culicoides odiatus* Austen, 1921 | | | | • | | | • | | | 12 | presence | 1 | large | birds |
| *Culicoides paolae* Boorman, Mellor & Scaramozzino, 1996 | | | | • | | • | • | | | 13 | presence | 1 | large | birds |
| *Culicoides (Monoculicoides) parroti* Kieffer, 1922 | | • | • | • | • | | • | • | | 4 | presence | 1 | small | indefinite |
| *Culicoides pictipennis* (Staeger, 1839) | | | | | | | • | | | 13 | presence | 1 | large | birds |
| *Culicoides poperinghensis* Goetghebuer, 1953 | | | | • | | | • | | | 6 | absence | 1 | small | mammals |
| *Culicoides pseudopallidus* Khalaf, 1961 | | | • | | • | | • | | | 13 | presence | 1 | large | birds |
| *Culicoides (Culicoides) pulicaris* (Linnaeus, 1758) | | • | • | • | • | • | • | • | • | 6 | absence | various | small | mammals |
| *Culicoides (Culicoides) punctatus* (Meigen, 1804) | • | • | • | • | • | • | • | • | • | 6 | absence | various | small | mammals |
| *Culicoides (Monoculicoides) puncticollis* (Becker, 1903) | | | | • | • | | • | | | 4 | presence | 1–2 | small | indefinite |
| *Culicoides (Pontoculicoides) saevus* Kieffer, 1922 | | • | | • | | | | | | 5 | presence | 1 | large | indefinite |
| *Culicoides (Oecacta) sahariensis* Kieffer, 1923 | | | • | • | • | • | • | • | | 6 | presence | 1 | large | indefinite |
| *Culicoides (Avaritia) scoticus* Downes & Kettle, 1952 | • | • | • | • | | • | • | • | • | 6 | absence | 1 | small | mammals |
| *Culicoides (Wirthomyia) segnis* Campbell & Pelham-Clinton, 1960 | | | | | | | • | | | 12 | presence | 1 | large | birds |
| *Culicoides shaklawensis* Khalaf, 1957 | | | | • | | | • | | | 6 | absence | 1 | large | indefinite |
| *Culicoides (Culicoides) subfagineus* Delécolle & Ortega, 1998 | | | | • | • | | • | | | 6 | absence | various | small | mammals |
| *Culicoides submaritimus* Dzhafarov, 1962 | | | | | | | | | • | 12 | presence | 1 | large | birds |
| *Culicoides univittatus* Vimmer, 1932 | | | | • | • | • | • | • | • | 13 | presence | 1 | large | birds |
| *Culicoides yemenensis* Boorman, 1989 | | | | • | • | | • | | | 8 | presence | 1 | large | birds |
| **Species richness** | 11 | 11 | 18 | 25 | 23 | 12 | 28 | 13 | 13 | | | | | |

1W, Proaza; 2W, R.N.C.Boumort; 3W, Puig la Penya; 4W, Quintos de Mora; 5W, La Morera; 6W, El Juanar; 7W, La Almoraima; 2D, Aramunt; 3D, Vilanova de la Muga. AF, antennal flagellomer; SC, sensilla coeloconica; SP, sensory pit.

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community patterns, according to its vector length and direction. The vector for annual precipitation divided localities in two dimensions, i.e. those located low and left-hand side of the graph, which had higher levels of annual precipitation, and those situated high and to the right-hand side. High and low temperatures also correlated with the ordination in a left-right dimension. The Pearson's correlation coefficient between the altitude and the given ordination axis is <0.2, there was therefore no relationship between the ordination of the sites and their altitude.

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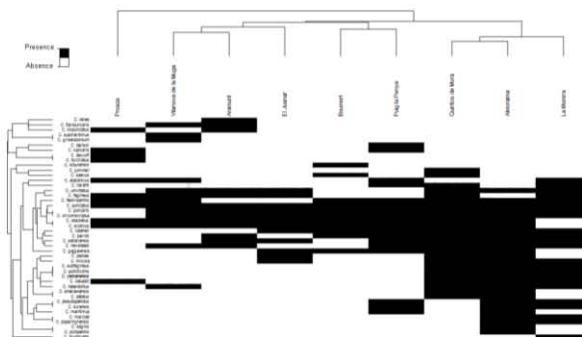
**Fig 1. Two-way cluster based on the Bray-Curtis similarity analysis of presence-absence data between *Culicoides* species and localities analyzed.**

doi:10.1371/journal.pone.0141667.g001

ANOSIM showed no significant differences neither between the type of ruminant (wild and domestic) sites nor among landscape features. Environmental variables appear as vectors that indicate relative correlation with MDS axes: LT (mean low temperature of the coldest month, in °C), HT (mean high temperature of the warmest month, in °C), AP (annual precipitation, in mm) and altitude (in meters).
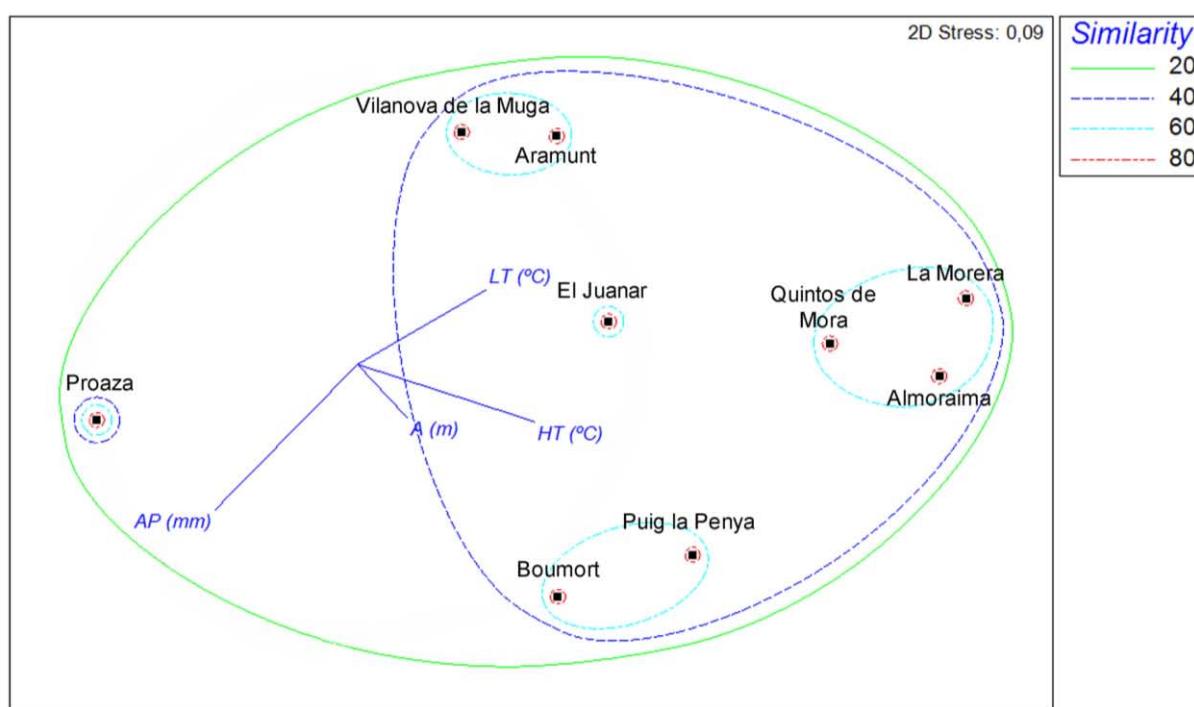


**Fig 2. Non-metric multidimensional scaling (MDS) ordination of Bray-Curtis similarity matrix for *Culicoides* communities based on presence-absence data.**

doi:10.1371/journal.pone.0141667.g002

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**Fig 3. Map showing the abundance of the main vector species of BTV and SBV in the sampling sites.**

Regarding the raw abundance (S1 Table), the 41.9% of specimens belonged to species with few or no spots on their wings. The rest of species, with spots on the wings, showed both a heterogeneous distribution and abundance. *Culicoides imicola* represented 15.4% of the overall total, 98.3% of which were found in La Almoraima. *Culicoides festivipennis* constituted 15.7% of the total captures, 79.7% of which were caught in Quintos de Mora. The Obsoletus group represented 6.6% of the captures, 54% of which were from Proaza and *C. circumscriptus* 9.4%, 70.8% of which were found in Quintos de Mora. Other less frequent detected species included: the Pulicaris group 1.5%, *C. newsteadi* 2.5%, the Similis group 3.4%, *C. punctatus* 1.2%, *C. parroti* also 1.2% and a final group including *C. impunctatus*, *C. puncticollis*, *C. shaklawensis*, *C. paolae*, the Odibilis group, *C. alazanicus*, the Fagineus group and the Sphagnumensis group 1.2%

At least one of the epidemiologically relevant species (the known BT vectors: *C. imicola*, and species of the Obsoletus and Pulicaris groups) was present at each sampling site (Table 2, Figs 3 and 4). The nMDS perfomed on the fourth root transformed abundances of epidemiologically relevant species based of the BC distance showed no significant differences between domestic and wild ruminant sites (ANOSIM, global $R = 0.119$, $p = 0.146$). *Culicoides imicola* was present at the four southern and central sites (4D-7D, 4W-7W), but absent from the other three north sites (1D-3D, 1W-3W, 2W; Table 2); the only exception was 2D, where a few females where unexpectedly trapped in July 2009 (Fig 5). This species was more abundant at livestock farms (4D-6D) than at corresponding areas with wild ruminants (4W-6W), although the pattern was reversed for site (7D and 7W; Table 2). During the study period (July 2009 to November 2010) *C. imicola* displayed a similar pattern in the southern and central sites, being detected from July to October (Fig 5). Species belonging to the Obsoletus group were found at all the sites except two (7D, 5W), being their captures anechdotically at 2W, 4D-6D (Table 2). This species was more abundant in areas where wild ruminants were present than on livestock farms. However, the geographic region 2 (2W-2D) was an exception to that, being the captures at 2D the most abundant of all sites, while anechdotically at 2W (Table 2). At northern sites their abundance was greater than in central and southern ones (Table 2). Species of the Obsoletus group, where present, displayed a similar activity pattern, being detected from June to November (Fig 5).

Species belonging to the Pulicaris group were trapped at all of the wild ruminant sites in medium levels of abundance (Table 2, Fig 3), while at livestock farms these species was either absent (4D-7D) or very scarce (1D-3D) with the exception of site 2D (Table 2). The Pulicaris group was active from June to November (Fig 5). When grouping sites according to ruminant type (wild or domestic), it was noted that the abundance of vector species was much higher at sites with wild ruminants (79.3%) than at livestock farms (20.7%) (Table 2).

*Culicoides* Species Associated with Wild Ruminants
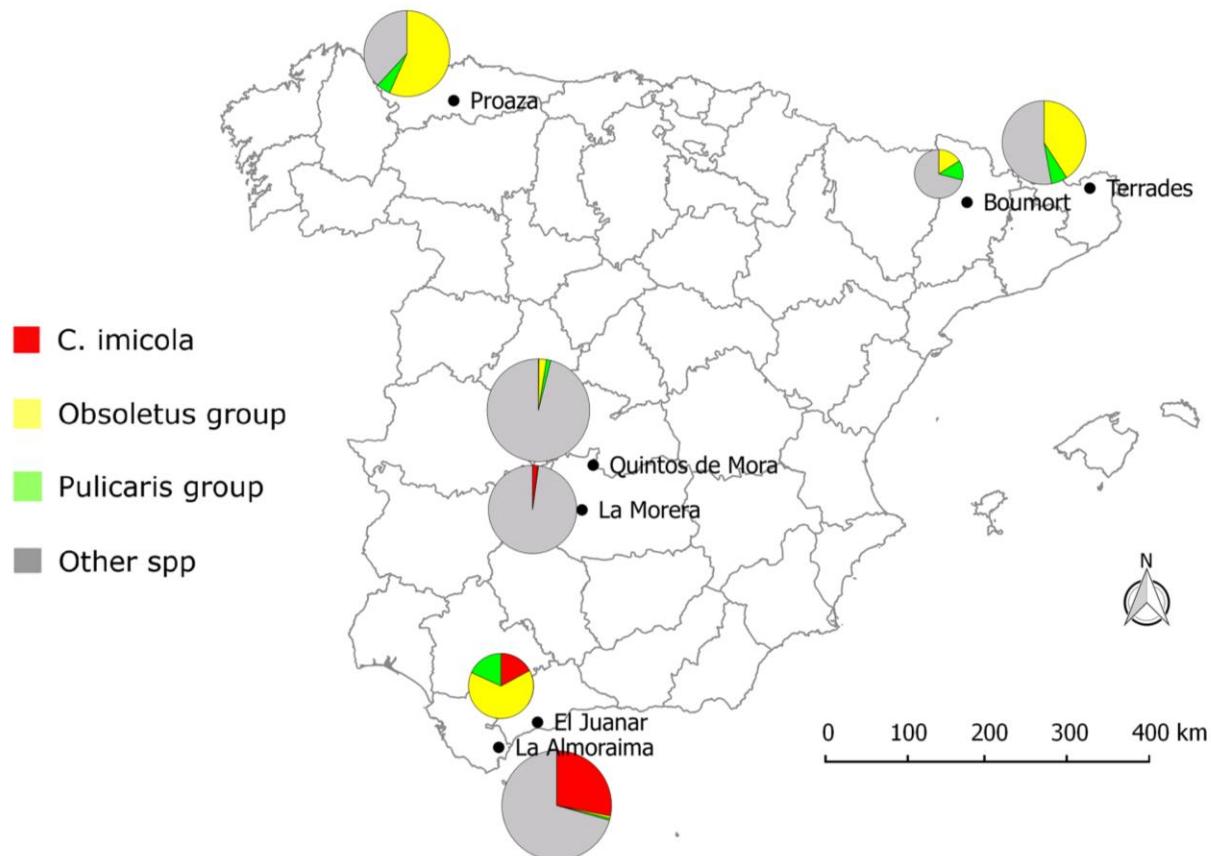


Fig 4. Map showing the relative abundance of the main vector species of BTV and SBV in the sampling sites.

doi:10.1371/journal.pone.0141667.g004

In the case of the inferred feeding habits of the different species of *Culicoides* at wild ruminant sites, 50% of the species found in our study were classified as bird-feeders (ornitophilic), 35% as mammal-feeders (mammalophilic) and 15% as indefinite, or with unclear host preference (Table 3). At livestock farms 2D and 3D, 41.2% of the species were ornitophilic, 47.0% were mammalophilic and 12.8% were indefinite (Table 3).

## Discussion

Few studies have been performed on *Culicoides* populations associated to natural areas with wild ruminants, and most of them are focused on parasites affecting wild bird populations [48–52]. The authors only found two studies that had been carried out in areas with wild ruminants, these had been conducted in Spain [53] and Nigeria [54].

Although a wide range of variation in the number of species present at different wild ruminant sites was detected, the results obtained in the present study showed that areas inhabited by wild ruminants tend to be very rich in *Culicoides* species (Table 3). It should also be noted that these values may have been underestimated, as diurnal species are not usually captured by

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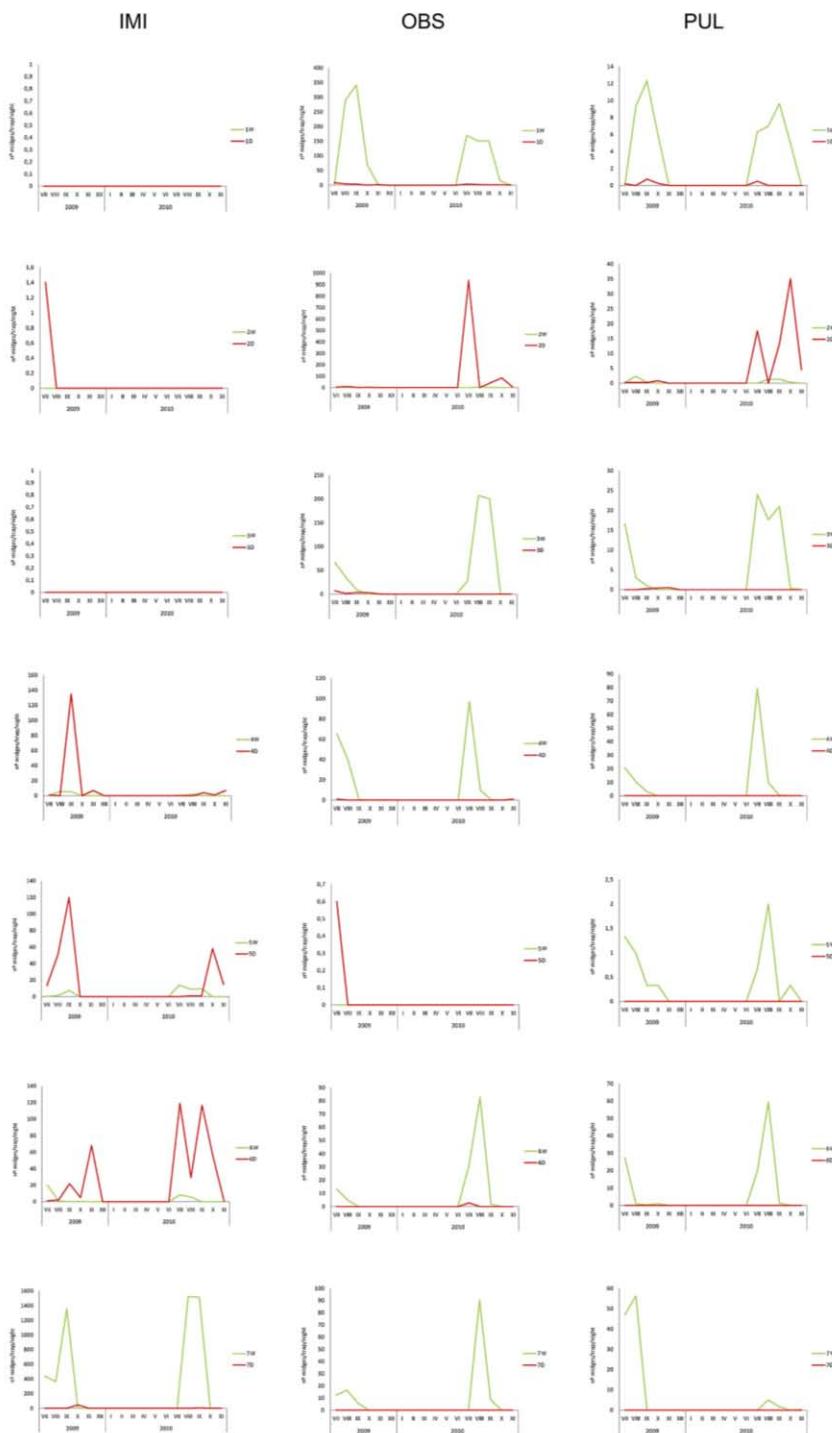
**Fig 5. Monthly n°midges/trap/night of the main vector species of BTV and SBV from July to December 2009 and from January to November 2010 at each wild and domestic ruminant sampling site.** 1W, Proaza; 2W, R.N.C. Boumort; 3W, Puig la Penya; 4W, Quintos de Mora; 5W, La Morera; 6W, El Juanar; 7W, La Almoraima; 1D, Tineo; 2D, Aramunt; 3D, Vilanova de la Muga; 4D, Piedrabuena; 5D, Navacerrada; 6D, Mijas; 7D, Castellar de la Frontera.

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CDC blacklight traps [55]. While Proaza (1W), R.N.C. Boumort (2W) and El Juanar (6W) had a relatively low number of species (S = 11–12), Quintos de Mora (4W), La Almoraima (7W) and La Morera (5W) had many (S = 22–28) (Table 3). Despite the variety of species identified it should be noted that all of the species detected in the present study had previously been recorded at livestock farms [47]. As can be seen from the MDS plot, Quintos de Mora, La Almoraima and La Morera grouped together (Fig 2). The two-way cluster suggests a group of species being exclusive from these southern and central localities, *C. subfagineus*, *C. yemenensis* and *C. puncticollis*. Proaza did not clustered with any other site, and was characterized by the presence of *C. dewulfi* and *C. furcillatus* and the absence of two otherwise widespread species *C. pulicaris* and *C. circmuscriptus*. The remaining sites are mostly characterized by the presence of the most common species (*C. obsoletus*, *C. scoticus*, *C. festivipennis*, *C. pulicaris* and *C. circumscriptus*) and the absence of the previously commented species. Although both of the two farms included in this analysis (Vilanova de la Muga and Aramunt) grouped together, the type of ruminant (wild vs domestic) had no influence on this grouping (Figs 1 and 2). In general, annual precipitation and the mean high temperature for the warmest month seemed to be the bioclimatic variables that most affected groupings, while the mean low temperature in the coldest month and altitude seemed to have a weak effect [56].

The distribution and relative abundance of epidemiologically relevant mammalophilic species (*C. imicola* and species belonging to the Obsoletus and Puliaris groups) at the different study sites (with wild ruminants) matched the known geographic pattern inferred from data obtained from the Spanish Bluetongue Entomological Surveillance Program (Table 2). *Culicoides imicola*, which is the main BTV vector in the Mediterranean Basin [3,57,58], was detected in the 4 southern and central areas, i.e. the warmest parts of Spain, but absent from the northern ones (Table 2, Figs 3 and 4). The large scale distribution pattern seems to be strongly influenced by the requirements of the species for high summer temperatures and dry summer conditions [59]. *Culicoides imicola* was more abundant at livestock farms than at natural areas with wild ruminants, with the exception of site 7W-7D (Table 2). Nevertheless, the activity patterns of the different species were similar at the central and southern sites. Interestingly, in areas with wild ruminants, *C. imicola* was active from July to September, whereas at the central and southern livestock farms, its activity continued until November (Fig 5). The Obsoletus group was present in all of the areas except in La Morera. However, it abundances were much greater at the northern than central or southern sites (with the exception of site 2D; Table 2). The activity pattern was homogeneous for all the natural areas with both wild ruminants and livestock farms, with the activity period being from July to October (and rarely until November, Fig 5). This distribution has been explained by the fact that species belonging to Obsoletus group requires areas with relatively low annual average $T^a$ and high soil moisture [60]. These results are in line with Calvete et al. [59], who described a similar latitudinal abundance pattern for livestock farms on the Iberian Peninsula. While *C. imicola* predominated in the warmest zones, species from the Obsoletus group predominated in those with relatively low mean annual temperatures. Although being located in the south, El Juanar had a species composition and relative vector abundances similar to northen localities. This pattern could have been influenced by bioclimatic values (Table 1), but also by other factors such as the abundance of suitable hosts and the presence of appropriate breeding sites [61]. In contrast to what

was observed for *C. imicola* and the Obsoletus group, the species belonging to the Pulicaris group were captured in all of the different natural areas with low to medium abundance values (Fig 2). Interestingly, such a pattern was not found for livestock farms, where the Pulicaris group was absent from all the central and southern farms (Table 2). At the sites in central Spain, none of the mammalophilic species stood out for its abundance; as a result, *C. imicola*, *C. punctatus* and species belonging to the Obsoletus and Pulicaris groups were trapped in similar (low) quantities (Table 2).

Important differences in the relative abundance of males and females were detected at the wild ruminant sites (S1 Table). The percentage of parous females captured was high (80%). Parous females are those that have completed at least one gonodotrophic cycle and which are already bloodfed and able to be infected if fed on a viraemic host. The active dispersal of adult midges belonging to the genus *Culicoides* is usually quite short, usually being limited to a few hundred metres from their breeding sites and at most to 2–3 km/day [62, 2], and only under very specific temperature, wind and humidity conditions they can become displaced over larger distances by wind [63, 64]. Since the livestock farms closest to the study sampling sites were at distances of between 1 and 10 km (Table 1), it could be assumed that most of the captured females that already had bloodmeal would have biten feral fauna. Regarding the feeding habits of the different species of *Culicoides*, it should be noted that the classification used in this work (Table 3) was based on morphological aspects [43] that were similar to those used in works that used molecular approaches to identify midge bloodmeals [65, 66]. In general, when comparing livestock farms, in natural areas with wild ruminants, it was possible to detect an increase in the relative abundance of ornitophilic species, such as *C. circumscriptus* and *C. festivipennis* (with these being most abundant at Quintos de Mora), and species with an unclear host preference, such as those belonging to the Similis group and *C. parroti*. Such an increase in abundance could be attributable to the greater variety of hosts and lower ruminant availability (density) to feed on in such natural areas [67, 68]. Until now, *Culicoides* species with ornitophilic and indefinite feeding habits had not been considered epidemiologically important for Bluetongue or Schmallenberg diseases. However, some studies have recently shown that *Culicoides* can be opportunists feeders, with species previously considered as ornitophilic or indefinite feeders have been detected feeding on mammals [69, 52, 70]. The fact that they represent 65% of the *Culicoides* caught in the wild ruminant areas, highlights the importance of conducting further studies to obtain more precise information about the feeding patterns of ornitophilic species and those with unclear feeding habits.

With regard the specific objectives of this study, our results showed: i) the composition of *Culicoides* species did not depend on the ruminant type present, ii) the main vector species for BTV and SBV present on the livestock farms were also present in neighbouring natural areas with wild ruminants, which would support their putative role as bridge vectors for the transmission of arboviruses between domestic and wild ruminants (in addition to their recognised role as epizootic vectors) and iii) the presence of non-vector *Culicoides* species in areas with wild ruminants that had previously been found in association with domestic ruminants, suggesting an irrelevant role in the maintenance of *Culicoides* transmitted arboviruses to wild ruminants in the region. Ornitophilic and indefinite species were more abundant in areas with wild ruminants than in those with livestock farms, with the abundance of mammalophilic species being reduced.

Overall, the present study would support the hypothesis that wild ruminant communities could serve as arbovirus reservoirs for *Culicoides* transmitted arboviruses. Wild ruminants are susceptible to various *Culicoides* transmitted viral diseases and our data confirmed that they are in close contact with major *Culicoides* vector species. Well known *Culicoides* vector species (*C. imicola* and Obsoletus group) could act as bridge vectors and circulate pathogens at the

interface between wild and domestic ruminant communities. Based on this hypothesis, the bypass of the pathogen among wild/domestic communities mediated by *Culicoides* bridge vectors (*C. imicola* and Obsoletus group) would facilitate the interseasonal BTV and SBV reintroduction among domestic ruminants. To further support the hypothesis, future studies will be needed to determine the bloodfeeding preferences of *Culicoides* in areas where wild ruminants are present.

## Supporting Information

**S1 Table. Species or species group abundance at each wild ruminanats sampling sites.** [71]. P, parous; N, nuliparous [34].
(XLSX)

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## Author Contributions

Conceived and designed the experiments: ST FMM NP. Performed the experiments: ST FMM MD AO AA FRF NP. Analyzed the data: ST FMM MV ASM NP. Contributed reagents/materials/analysis tools: RE. Wrote the paper: ST FMM ASM NP.

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**Capítol IV**

**Abstract**

Many species of genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) are vectors of pathogens as Bluetongue virus (BTV) and the recently discovered Schmallenberg virus (SBV), both cause important infectious, non-contagious, insect-borne viral diseases which affected domestic and wild ruminants. The role of wild ruminants in relation to disease overwintering and transfer to farms with domestic ruminants is poorly studied. In this context, the knowledge of *Culicoides* species feeding behavior is an important parameter to better understand the epidemiology of Bluetongue and Schmallenberg diseases.

Samplings were conducted in Spain, in areas inhabited by different wild ruminant species during midge peak activity season (summer and autumn), 264 blood engorged *Culicoides* females were trapped, including major BTVand SBV vector species. Blood meal origin was identified from 114 females of 14 species. Most *Culicoides* feed on mammals (91.1%) and the rest (8.9%) on birds. The most abundant host identified was red deer (66.7%) followed by human (13%) and fallow deer (6.1%). Eleven of fed *Culicoides* species have fed on mammalian hosts, of which 5 species are considered BTV and /or SBV vectors (*C. obsoletus*, *C. scoticus*, *C. imicola*, *C. pulicaris* and *C. punctatus*), also found species previously described as ornitophilic or indefinite feeding habits as *C. yemenensis*, *C. parroti* *C. saevus* and *C. univitattus*. Two of *Culicoides* species have fed exclusively of avian hosts (*C. cataneii* and *C. circumscriptus*) and one species (*C. festivipennis*) fed both avian and mammals hosts.

**Key words:** *Culicoides*, wild ruminants, host preference, BTV, SBV, vector, Spain

**Introduction**

The genus *Culicoides* has been extensively studied for having species that act as vectors of important diseases. There are approximately 1400 *Culicoides* species in the world [1, 2]. The 96% of females are expected to be obligate blood-feeders. *Culicoides* can act as vector of protozoa, filarial worms and viruses affecting humans and animals, both mammals and birds [3]. One of the most economically important pathogen transmitted by *Culicoides* is BTV. This virus is a double stranded RNA virus of the genus *Orbivirus* that

produce an infectious non-contagious disease of domestic and wild ruminants [4]. In Europe, Bluetongue (BT) disease is widely transmitted by *Culicoides* species in the Obsoletus group, and *C. imicola* in Mediterranenan basin. *Culicoides* transmit Schmallenberg (SB) disease to ruminants as well. SB is produced by a virus of the Orthobunyavirus genus, family Bunyaviridae and Simbu serogroup. SB was detected for the first time in Germany and in the Netherlands, in summer and autumn 2011 [5-9]. Since then, the disease has detect almost the whole Europe and its presence was confirmed in Spain (March 2012) affecting sheep and goats in the south of the country. Up to date, eight species of *Culicoides* have been described as vectors for SBV in Europe: *C. obsoletus*, *C. scoticus*, *C. dewulfi*, *C. chiopterus* [5-7], *C. punctatus* [8], *C. pulicaris, C. nubeculosus* and *C. imicola* [9], all of them considered as vectors of BTV with the exception of *C. punctatus* and *C. nubeculosus.* Although a recent study indicates that *C. newsteadi* and *C. punctatus* might act as BTV vectors [10].

Wildlife may play an important role in the transmission of BT and SB as suggested by the high seroprevalence of BTV and SBV antibodies found in wild ruminant species in Europe [11, 12]. The standing *Culicoides* host-preferences are a key issue to better understand the epidemiology of different diseases transmitted by this genus. Some studies performed inferences on *Culicoides* host preferences based on morphological analysis of sensory structures such as antennae and palps [13]. More precise studies used engorged females with a recent bloodmeal in their abdomen to identify the blood source and establish the feeding pattern of *Culicoides* species. Early studies on *Culicoides* feeding pattern were based on serological techniques [14-17] and immunological assays [18, 19]. However, these methods allowed the identification of a limited number of vertebrate species. The use of molecular techniques increased the accuracy of host identification at the species level [20, 21]. Most studies dealing with *Culicoides* host feeding preferences have been conducted on *Culicoides* collected around livestock areas. Nevertheless, few of them have been performed in natural ecosytems targeting wild hosts [22, 23].

In Spain, *Culicoides* were described to be abundant and in close contact with wild ruminants in natural ecosystems [24]. The mechanism beyond the spillover of BT and SB from domestic to wild ruminants (and vice-versa) is poorly known. Therefore, to determine the *Culicoides* host-feeding preferences in natural ecosystems is mandatory to understand

such mechanisms. This information will led to propose the *Culicoides* species that can act as bridge and enzootic vectors for BT and SB.

## Materials and Methods

### Sampling

*Culicoides* specimens identified in the study were trapped between years 2009-2010, in seven Spanish private areas characterized by their distinctive bioclimatic features and wild ruminant communities (Fig 1; S1 Table). Data relating to bioclimatic variables and altitude were obtained from the climatic atlas of the Iberian Peninsula [25]; landscape variables were obtained from the Global Environment Monitoring database [26], and the distribution of the different ruminants within Spain was obtained from an atlas of land mammals in Spain [27]. Three CDC traps (John W. Hock Company, Gainesville, FL, USA), maximum 15 meters separated, were placed closely water points used as troughs for wild ruminants, and a minimum distance of 1 kilometer from domestic ruminants; being switched on at dusk an off at dawn the following day for three consecutive nigths once a month, during the months of summer and autumn of two consecutive years. In Spain seven different species of wild ruminants are present, red deer (*Cervus elaphus*) being the most abundant, fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), mouflon (*Ovisaries musimon*), Spanish ibex (*Capra pyrenaica hispanica*), Pyrenean chamois (*Rupicapra pyrenaica*) and aoudad (*Ammotragus lervia*).
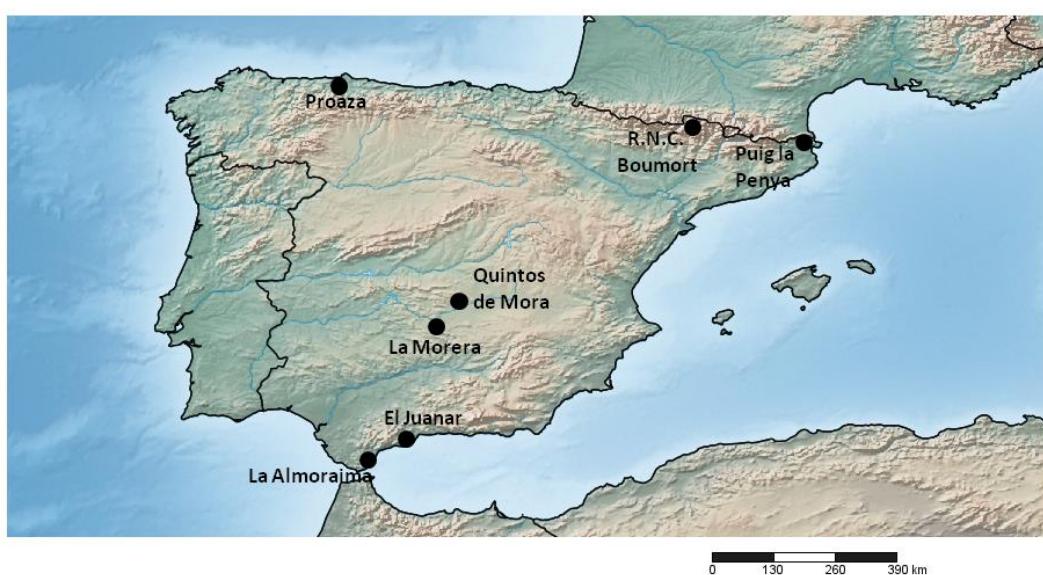


**Figure 1.** Map with sampling sites

**Morphological and molecular identification**

Trapped insects were killed by drowning in the capture boat, which contains water with soap, and stored in ethanol 70% for further identification. *Culicoides* midges were first identified under a stereomicroscope (Nikon SMZ) by morphology at species-group level according to wing pattern. An accurate morphological identification was performed of at least one individual of each sex of all species cited in the present work, were dissected using sterilized ultrafine tweezers, mounted on slides with Canada Balsam solution, by examination using a Nikon Eclipse E200 light microscope. We used different taxonomic keys [28-32]. In order to confirm the presence of Obsoletus group females, precise identification of females of the Obsoletus group was performed by means of PCR according to the procedures described in 33 and 34.

**Blood identification**

Blood engorged females were used for DNA extraction, amplification and sequencing of a fragment of the subunit I of the cytochrome oxidase gene (COI) as described in 35.

**Results**

**Collection of biting midges**

The amount of *Culicoides* collected was 102693 specimens [24]. Less than 1% of collected *Culicoides* were blood engorged females (n=264). Identification of host source based on genetic blood-meal characterization was successful in 114 (44%) engorged females. Moreover, engorged females with positive host identification belonged to 14 *Culicoides* species. *C. obsoletus* was the most abundant species (n=47), followed by *C. scoticus* (n=13), *C. punctatus* (n=10) and *C. imicola* (n=9) all of them known vectors of BTV and/or SBV (Table 1). Engorged females were collected at all sampling sites except in Boumort where collections were scarce (0.1% total captures [24]; S1 Table).

**Table 1.** Blood meal sources (vertebrates hosts) of engorged *Culicoides*. **Pro.,** Proaza; **P.P.,** Puig la Penya; **Q.M.,** Quintos de Mora; **L.M.,** La Morera; **E.J.,** El Juanar; **L.A.,** La Almoraima.

| | Mammals | | | | | | Birds | | | | | | |
| Species | Capra pyrenaica | Cervus elaphus | Dama dama | Homo sapiens | Sus scrofa | Bos taurus | Cyanistes caeruleus | Delichon urbica | Erithacus rubecula | Sylvia cantillans | Turdus merula | Upupa epops | % Blood engorgrd |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. cataneii* | | | | | | | L.A.**1** | | | | | L.M.**1** | 1.8 |
| *C. circumscriptus* | | | | | | | | | | | Q.M.**1** | L.A.**1** | 1.8 |
| *C. festivipennis* | | | | L.M.**1** | | | | Pro.**1** | Q.M.**1** | Q.M.**1** | Q.M.**2** L.A.**1** | | 6.1 |
| *C. imicola* | | L.A. **6** | | | | L.A. **3** | | | | | | | 7.9 |
| *C. newsteadi* | | L.A. **1** | L.A. **2** | L.A. **1** | | L.A. **1** | | | | | | | 4.4 |
| *C. obsoletus* | E.J. **1** | Pro. **26** P.P.**10** Q.M. **2** L.A **4** | | Pro. **1** Q.M. **2** | Pro. **1** | | | | | | | | 41.1 |
| *C. parroti* | | Q.M. **3** L.A. **1** | L.A.**1** | | | | | | | | | | 4.4 |
| *C. pulicaris* | | P.P. **2** | P.P. **1** | Pro.**1** | | | | | | | | | 3.5 |
| *C. punctatus* | | P.P. **2** Q.M. **6** | P.P. **2** | | | | | | | | | | 8.8 |
| *C. saevus* | | Q.M. **2** | | | | | | | | | | | 1.8 |
| *C. scoticus* | | Pro. **4** P.P.**1** L.A.**4** | P.P.**1** | L.A. **3** | | | | | | | | | 11.3 |
| *C. subfagineus* | | L.A. **1** | | | | | | | | | | | 0.9 |
| *C. univitattus* | | | | L.A. **5** | | | | | | | | | 4.4 |
| *C. yemenensis* | | L.A. **1** | | L.A. **1** | | | | | | | | | 1.8 |
| **% Blood engorged Culicoides** | **0.9** | **66.7** | **6.1** | **13** | **0.9** | **3.5** | **0.9** | **0.9** | **0.9** | **0.9** | **3.5** | **1.8** | |

**Blood host identification**

Eleven out of 14 *Culicoides* species took a blood meal exclusively on mammalian hosts, including representatives of major BTV and/or SBV vectors (*C. obsoletus*, *C. scoticus*, *C. imicola*, *C. pulicaris and C. punctatus*), and one specie described as ornitophilic [24] fed just on human (*C. univitattus*). Two out of 14 *Culicoides* species fed exclusively on avian hosts (*C. cataneii* and *C. circumscriptus*). A single species, *C. festivipennis*, was confirmed to fed on mammals and birds (Table 1, Fig 2). The 83.5 % of blood engorged females analyzed been fed wildlife, 13% human and 3.5% cattle. In all areas studied human presence is frequent but not abundant, explainig the 13% of *Culicoides* fed human blood. All females fed cow were caught in the same area (La Almoraima) one kilometer from the closest domestic ruminant area (S1 Table).

*Culicoides* fed on 12 host species, 6 mammals and 6 birds. However 91% of the bloodmeals identified were of mammals and 9% of birds (Table 1). In blood-meals, the most frequently identified host was red deer (*Cervus elaphus,* 66.7%), human (*Homo sapiens,* 13%) and fallow deer (*Dama dama,* 6.1%). The most frequent avian host was blackbird (*Turdus merula,* 3.5%) (Table 1, Fig 2).
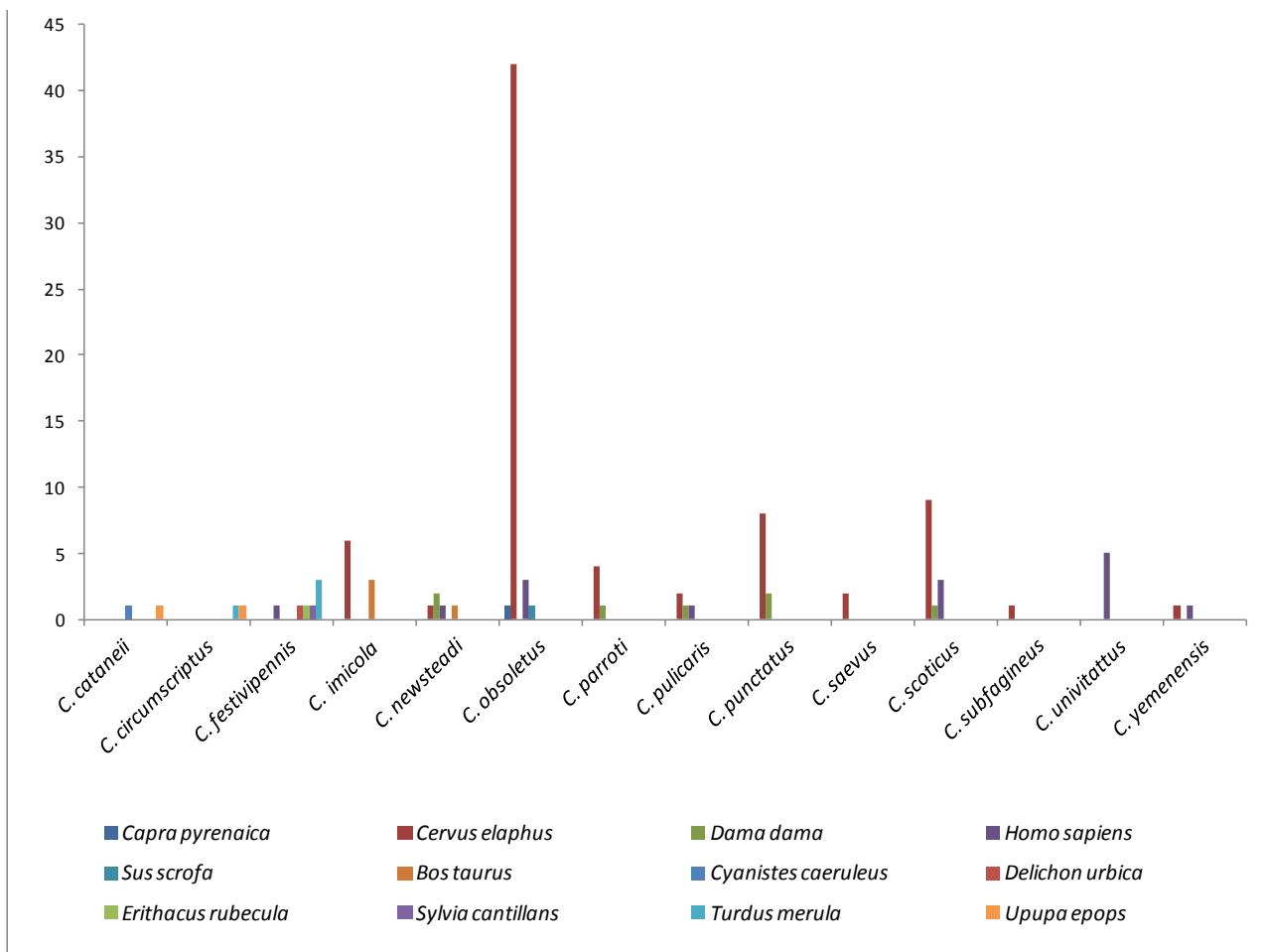


**Figure 2**. *Culicoides* species host preferences

Most abundant wild ruminant of each zone was detected as the prefered host for *Culicoides* except in La Morera (Fig 3). In all areas except El Juanar the most abundant host was red deer (*Cervus elaphus* ), and in El Juanar was Spanish ibex  (*Capra pyrenaica hispanica*) (S1 Table). In La Morera red deer was also the main wild ruminant, however we could only detect blood of  human and  bird (Table 1, Fig 3).
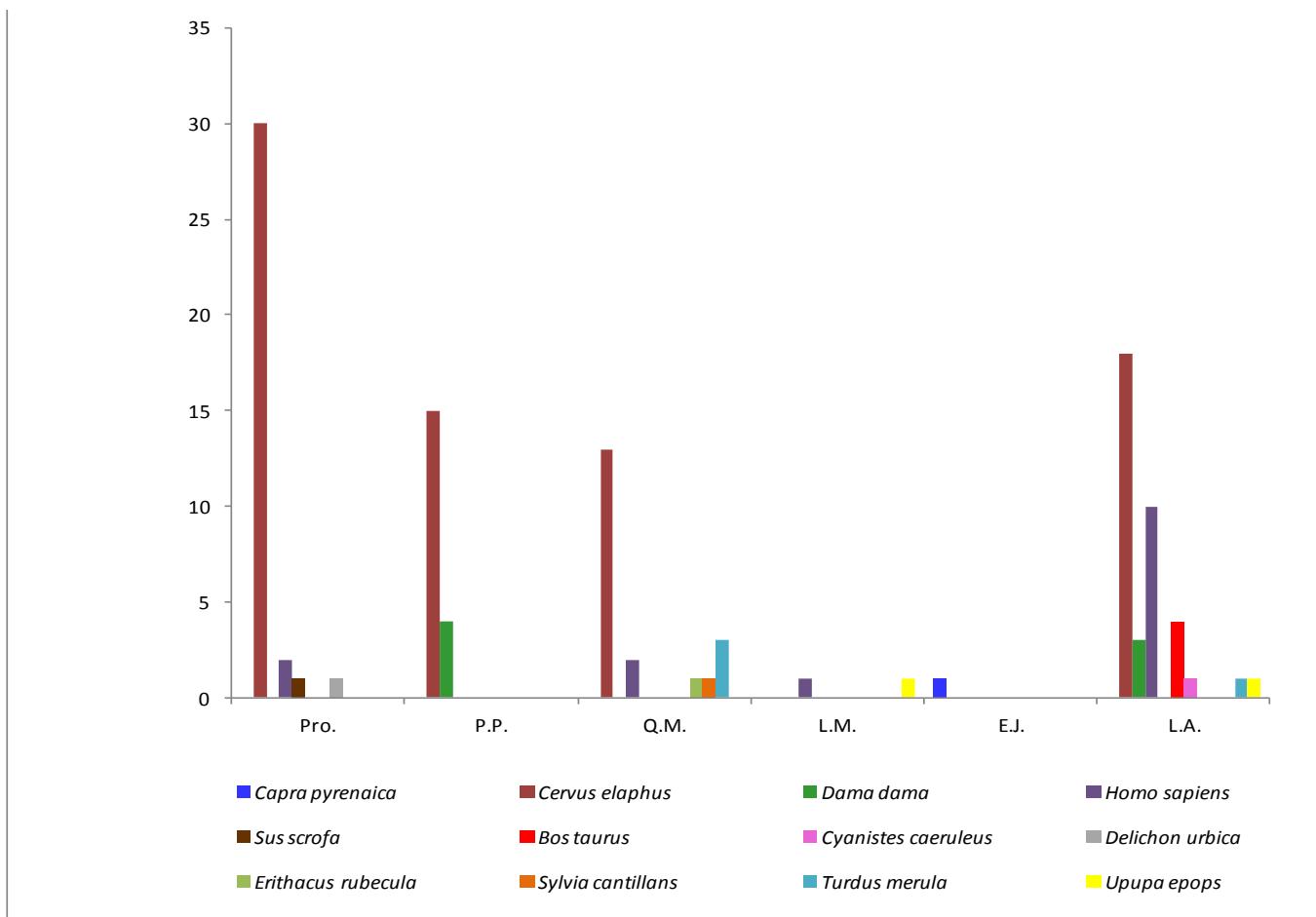


**Figure 3.** *Culicoides* host preferences in each sampling sites**. Pro.,**Proaza;  **P.P.,** Puig la Penya; **Q.M.,** Quintos de Mora; **L.M.,** La Morera; **E.J.,** El Juanar; **L.A.,** La Almoraima

**Discussion**

Recent studies highlight that most *Culicoides* species are able to feed on several vertebrate species, but present clear preferences for mammals or birds [20].The Knowledge on vector host preferences is critical to better understand the epidemiology of different diseases [36, 37]. The *Culicoides* host preferences are dependent on the intrinsic features of each *Culicoides* species and on the environmental factors as well, such as host availability [37, 38]. Opportunistic or selective host tendencies of a vector may affect the spread of disease [38].

Most studies of *Culicoides* host preferences focused on mammalophilic species, whereas ornithophilic species received less attention [3, 39, 40]. In addition, these studies were usually performed in farms or rural areas with livestock because of the economical importance of BT for livestock production [41-47].

Studies on host feeding preferences have demonstrated that many species of *Culicoides* are not generalist and feed specifically either on birds or mammals [48], the results obtained support the former statement. Nevertheless *Culicoides* not appear to have host species specificity beyond the separation of mammalophilic and ornithophilic species, they feed opportunistically on host species. Host selection generally seems to reflect host availability [45].

*Culicoides* species collected at natural ecosystems with wild ruminants feed on mammals and birds. However blood engorged females of *Culicoides* species previously described as strict mammalophilic bloodfeeders [24] contained blood of mammalian origin. The ones described before as ornitophlic feeders contained blood of avian origin except *C. festivipennis* and *C. univitattus,* which have been found feeding on mammals, specifically on humans. These results are in accordance with other studies that described C*. festivipennis* as generalist host specie [48]*. Culicoides* species with generalist preferences  are of special interest because they could facilitate the emergence of new diseases [48].

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Four new *Culicoides* hosts were described: two wild ruminants, fallow deer (*Dama dama)* and Spanish ibex (*Capra pyrenaica)*, and two birds, *Sylvia cantillans* and *Upupa epops*. Besides that the main BT and SB vectors (*C. imicola*, *C. obsoletus*, *C. scoticus*, *C. pulicaris* and *C. punctatus*) collected in the study fed exclusively on mammals , mainly wild ruminants (70/82; Table 1). The present study reports the host origin of *C. imicola* bloodmeals for the first time and its description feeding on wild ruminant in Europe. In Africa *C. imicola* was described feeding mainly on horses, cattle and sheep [49], and also on humans, birds and dogs [21]. The results of present study confirm the hypothesis that *C. imicola*, *C. obsoletus*, *C. scoticus*, *C. pulicaris* and *C. punctatus* can act as bridge vectors and circulate pathogens at the interface between wild and domestic ruminant communities.

There are some species with unclear or ornitophilic feeding habits [24] as *C. parroti*, *C. saevus* and *C. yemenensis,* with low catches, but feeding exclusively on wild ruminants in natural ecosystems sampled*.* These species are poorly studied so far because they are scarcely collected in domestic ruminant farms. For some years, different authors pointed that UV light-traps does not accurately reflect proportions of *Culicoides* biting in the field [49] Som estudies comparing animal-baited traps and UV-light/suction traps suggested the later traps could understimate the assessment of BTV risk [50,51].

*C. parroti*, described as having unclear feeding habits [24] and seems to be present at farms feeding on mammals but miss-collected [51]. The species belongs to the subgenus *Monoculicoides*, that includes the main BT vectors in the US (*C. sonorensis* C. *variipennis* and *C. occidentalis*), and has a wide distribution, through Europe to Russia. Thus, it's potential role on the epidemiology of BTV and SBV should be further investigated.

The 13% of engorged *Culicoides* bite on humans, which is in accordance previous studies that found the common mammalophilic *Culicoides* species in Europe, including arboviruses vectors, occasionally been recorded biting humans [21, 43, 48, 52]. Attacks on humans mainly occur when alternative host as livestock or wildlife are either scarce or absent. Worldwide, the role of *Culicoides* in the transmission of zoonotic arboviruses is currently unclear, but thought to be limited [52].

To conclude, *Culicoides* species collected in natural ecosystems inhabited by wild ruminants are mostly mammalophilic and bite preferably on wild ruminants. The main BT and SB vector species in Europe fed on wild ruminants as well. Seroprevalence studies and experimental infections confirmed wild ruminants are susceptible to BTV and SBV infection [11, 12, 53-60]. We therefore suggested the putative vectors of BT and SB can act as bridge vectors for the spillover of both diseases between domestic and wild ruminants.

## Acknowledgements

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**S1 Table.** Data summary of ecological variables and characterization of the sampling sites [22]. **A,** altitude; **AP,** annual precipitation; **LT,** mean low temperature of the coldest month; **HT,** mean high temperature of warmest month[23,24].

| Sampling site | Geographical variables Coordinates | Bioclimatic Classification | A(m) | HT(°C) | LT(°C) | AP(mm) | Domestic ruminants (distance in km) | Water (distance in meters) | Ruminants in sampling place (more abundant - less abundant) | Blood engorged Culicoides % total |
|---|---|---|---|---|---|---|---|---|---|---|
| Proaza | 43°12' N 06°03' W | Temperate oceanic submediterranean | 349 | 25 | 0 | 1000 | Farm (4.5) | wet soil, not water on surface | red deer, roe deer | 29.8 |
| R.N.C.Boumort | 42°12' N 01°06' E | Temperate hyperoceanic | 1276 | 25 | -7.5 | 900 | Free domestic livestock (1) | Pond (<5) | red deer, fallow deer, roe deer, chamois | 0 |
| Puig la Penya | 42°31' N 02°32' E | Mediterranean pluviseasonal oceanic | 228 | 27.5 | 2.5 | 1000 | Farm (2) | Pond (25) | red deer, fallow deer, mouflon | 16.7 |
| Quintos de Mora | 39°23' N 04°06' W | Mediterranean pluviseasonal oceanic | 718 | 35 | 0 | 482 | Farm (10) | Pond (<5) | red deer, fallow deer, roe deer | 17.6 |
| La Morera | 38°54' N 04°16' W | Mediterranean pluviseasonal oceanic | 707 | 35 | 2.5 | 500 | Farm (4) | Pond (5) | red deer, mouflon, aoudad | 1.8 |
| El Juanar | 36°34' N 04°53' W | Mediterranean pluviseasonal oceanic | 870 | 27.5 | 2.5-5 | 850 | Farm (5) | Cement trough (<5) | spanish ibex | 0.88 |
| La Almoraima | 36°27' N 05°43' W | Mediterranean pluviseasonal oceanic | 45 | 30.5 | 7.5 | 955 | Farm (4) | wet soil, not water on surface | red deer, fallow deer, roe deer, mouflon | 33.3 |

**Discussió**

## Discussió

El gènere *Culicoides* és un grup d'insectes complex a nivell taxonòmic. Això es deu a que trobem espècies filogenèticament molt properes, conegudes com espècies críptiques, que resulten molt difícils d'identificar morfològicament (Campbell i Pelham-Clinton, 1960). La identificació, diagnòstic i descripció de les espècies críptiques de *Culicoides* ha millorat considerablement en els darrers anys gràcies a eines recents com la biologia molecular (Gomulski *et al*., 2006; Pagès *et al*., 2009; Lassen *et al*., 2011; Sarvasova *et al*., 2014) i la morfometria geomètrica (Muñoz-Muñoz *et al*., 2011; Muñoz-Muñoz *et al*., 2014). Fet que provoca que en els últims anys estiguin identificant-se noves espècies dins aquest gènere (Ramilo *et al*., 2013; Nielsen i Kristensen, 2015).

L'interés envers el seu estudi ha estat recent, tot i que desde els anys 50 algunes espècies de *Culicoides* han estat conegudes per produir grans molèsties a nivell mundial a humans i bestiar a través de la seva picada (Cilek *et al*., 2003; Mands *et al*., 2004; Carpenter *et al*., 2006). No obstant, no ha estat fins els últims anys quan els *Culicoides* han adquirit gran importància com a transmissors de diferents malalties, principalment en el camp de la sanitat animal.  Quan la malaltia de la LB es va estendre i aparèixer de manera recurrent fora dels seus límits de distribució habituals, l'estudi de la LB va centrar l'interés en el coneixement del gènere *Culicoides* (Roy, 1992; Purse *et al*., 2005; Wilson i Mellor 2008; Purse *et al*., 2008). El canvi en la distribució de la LB es creu que és degut en gran part a l'augment de les temperatures causat pel canvi climàtic (Wittman *et al.*, 2001; Purse *et al.*, 2005) que hauria provocat un canvi en la distribució i capacitat vectorial de les espècies de *Culicoides,* (Gerry *et al*., 2001; Purse *et al*., 2005; Purse *et al*., 2008; Wilson i Mellor, 2008; Gould i Higgs, 2009), afavorint la seva adaptació a zones tradicionalment més fredes (Purse *et al*., 2005; Hoffmann *et al*., 2008).  A aquest factor es va afegir molt recentment l'aparició a Europa (Alemanya, 2011) del virus SB (Hoffman *et al*., 2012). Fets que demostren per tant que hi ha molts aspectes de l'epidemiologia d'aquestes dues malalties transmeses pels *Culicoides* encara  desconeguts.

En tota malaltia de transmissió vectorial identifiquem tres factors primaris, el patogen, l'hoste i el vector. Les malalties de la LB i SB no en són una excepció. El patró i intensitat de la malaltia estan condicionats per les interaccions, sovint complexes i dinàmiques, entre

virus, hoste i *Culicoides*. El present treball contribueix en la millora del coneixement dels *Culicoides* amb contribucions importants en dues disciplines, la taxonomia i l'epidemiologia. Els dos primers estudis de la tesi profunditzen en la taxonomia de les espècies del gènere *Culicoides* a Espanya i en la descripció taxonòmica de dues noves espècies de *Culicoides* dins el subgènere *Culicoides*, on hi ha espècies de gran importància veterinària. Els dos darrers estudis de la tesi profunditzen en aspectes concrets de l'epidemiologia de malalties transmeses per *Culicoides*, en particular la LB i SB. Avaluant el paper que els remugants salvatges poden tenir a Espanya com a reservori d'aquestes malalties i com a potencial origen per la reintroducció de la malaltia en remugants domèstics. D'aquesta manera es van caracteritzar les poblacions de *Culicoides* presents en zones de remugants salvatges, i determinar les seves preferències d'hoste. Així doncs, el fil conductor de la tesi doctoral i els quatre estudis que comprèn són les espècies de *Culicoides* vectores de les malalties de la LB i SB.

El gènere *Culicoides* és molt extens, s'estima que està format per més de 1.400 espècies (Borkent i Wirth, 1997 actualitzat 2012). A España s'han identificat 81 espècies (Alarcón-Elbal i Lucientes, 2012), 45 de les quals gràcies al treball conjunt entre taxonomia clàssica i l'estudi genètic realitzat en la present tesi han pogut ser identificades a Catalunya en contacte amb remugants domèstics. En el primer treball realitzat (capítol I), la identificació de 45 espècies de *Culicoides* incrementa en 13 el nombre d'espècies identificades respecte la darrera actualització (Sarto i Monteys *et al.,* 2009). En aquest treball cal destacar la detecció i identificació de les espècies *C. yemenensis* Boorman (1989) com a nova cita per Europa, *C. coluzzii* Callot *et al.* (1970) i *C. sejfadinei* Dzhafarov (1958) com a noves cites per la Península Ibèrica i *C. pseudopallidus* Khalaf (1961) com a nova cita per Espanya.

Entre totes les espècies identificades a Catalunya basant-nos en la seva distribució al llarg de la zona d'estudi es van poder identificar diferents grups, els anàlisis van demostrar que aquesta distribució responia a dues variables climàtiques, la mitjana de les temperatures més altes del mes més calurós i la taxa de precipitació anual.

La taxonomia clàssica es veu molt reforçada quan es combina amb tècniques moleculars. Ha estat aquesta combinació la que ha permès millorar en la correcta identificació de les espècies críptiques existents dins el gènere *Culicoides*. Això resulta de

gran importància en aquells subgèneres on hi ha espècies que són vectores de malalties. Per exemple, la presència no detectada d'espècies críptiques pot causar gran heterogeneïtat en el grau de competència vectorial d'una espècie en diferents zones geogràfiques. Aquest va ser el cas de poblacions de les espècies *C. pulicaris* i *C. punctatus* del Regne Unit enfront el virus de la Llengua Blava (Carpenter *et al*., 2006).

El subgènere *Culicoides* està format per 59 espècies entre les que es troben les espècies *C. pulicaris*, *C. punctatus* i *C. newsteadi* reconeguts vectors de les malalties de la LB i/o SB (Balenghien *et al*., 2014; Goffredo *et al*., 2015). En conseqüència, les tres espècies estan incloses en els Plans de vigilància entomològica de la Llengua Blava que es duen a terme a diferents països de la Unió Europea. L'estudi exhaustiu dels individus semblants als de l'espècie *C. pulicaris* i als individus amb característiques morfològiques intermèdies entre *C. pulicaris* i *C. punctatus* ha permès la identificació de dues noves espècies críptiques *C. cryptipulicaris* i *C. quasipulicaris*. Amb aquestes dues noves espècies descrites s'incrementa a 61 el nombre d'espècies que actualment formen el subgènere *Culicoides*. El nombre d'espècies dins el subgènere *Culicoides* ja havia augmentat recentment (Nielsen i Kristensen, 2015), i es preveu que s'incrementi ja que segueixen sent molts els treballs amb informació per la potencial descripció de noves espècies críptiques (Meiswinkel *et al*., 2004; Gomulski *et al*., 2006; Pagès *et al*., 2009; Muñoz-Muñoz *et al*., 2011; Sarvasova *et al*., 2014;). Aquest fet també succeeix en altres subgèneres (Meiswinkel *et al*., 2015).

Les dades obtingudes en aquest estudi (Capítol II) ens han permès construir una clau dicotòmica amb caràcters morfològics qualitatius. Amb aquesta nova clau dicotòmica és possible identificar la majoria d'espècies a nivell mundial que són similars a *C. pulicaris* i *C. punctatus* excepte les femelles de la espècies *C. pulicaris, C. cryptipulicaris* i *C.neopulicaris,* i les espècies *C.almeidae* i *C. yukonensis,* que degut a una descripció original considerada incompleta no es van incloure a la clau.

Disposar d'eines com una clau dicotòmica amb caràcters morfològics no mètrics resulta indispensable per poder desembolicar l'entramat d'espècies críptiques. La diferència entre les claus dicotòmiques amb caràcters mètrics i les formades per caràcters no mètrics és la versatilitat i validesa dels darrers caràcters arreu del món. Els caràcters morfològics qualitatius (no mètrics) no estan condicionats per diferències en les condicions climàtiques entre diferents regions del món. Els caràcters mètrics tot i que poden

presentar diferències entre les espècies i ser vàlids per identificar-les en una zona concreta no són representatius de tot el rang de varietat intraespecífica, perdent per tant el seu valor quan es vol comparar amb altres regions, ja que s'ha vist que la mida dels insectes pot estar condicionada per diferents factors com la temperatura (Birdsall *et al*., 2000; Smith i Mullens, 2003; Muñoz-Muñoz *et al*., 2011), la concentració d'aliment i la densitat larvària (Jirakanjanakit *et al*., 2007; Lane, 1981; Smith i Mullens, 2003).

En el cas concret de les malalties de la LB i SB s'ha realitzat un gran nombre d' estudis tant en vectors (*Culicoides*) com en hostes susceptibles (remugants). El fet que siguin malalties que provoquen grans pèrdues econòmiques relacionades amb el bestiar domèstic ha fet que la gran majoria d'estudis s'hagin focalitzat en àrees de producció ramadera (remugants domèstics). En els darrers anys s'han fet grans avenços i adquirit nous coneixements en aquest terreny però tot i això la malaltia de la LB segueix apareixent allà on es creia controlada (BVA, 2015; Niedbalski, 2015). Arrel de fets com aquests van sorgir dubtes sobre quin paper podien estar jugant els remugants salvatges en la reintroducció de la malaltia en zones declarades prèviament com a lliures de la malaltia (Niedbalski, 2015). El tercer i quart estudi de la tesi (Capítols III i IV) ofereixen resultats que contribueixen a entendre aquesta qüestió.

Estudis previs demostren que els remugants salvatges són susceptibles a infectar-se amb les malalties de la LB i SB. Això s'evidencia per la presència d'anticossos específics per ambdós virus en estudis de seroprevalença en remugants salvatges (Ruiz-Fons *et al*., 2008; García *et al*., 2009; Rodríguez-Sánchez *et al*., 2010; García *et al*., 2011; Ruiz-Fons *et al*., 2014; Linden *et al*., 2012; Larska *et al*., 2014; Chiari *et al*., 2014; Laloy *et al*., 2014 Fernández- Aguilar *et al*., 2014). Hi ha una sèrie de factors que es donen en els remugants salvatges que suggereixen que aquests podrien tenir un rol important en l'epidemiologia d'aquestes dues malalties: i) una virèmia de llarga durada en el cas de la LB (López-Olvera *et al*., 2010), ii) les dues malalties solen passar desapercebudes per infeccions subclíniques en la majoria d'espècies (Falconi *et al*., 2011; Chiari *et al*., 2014; Laloy *et al*., 2014; Fernández-Aguilar *et al*., 2014), i iii) es tracta d'animals lliures que poden afavorir l'entrada i difusió de la malaltia, dels que és molt difícil tenir control del cens, moviment i estatus sanitari de tots els individus per falta d'informació i maneig, tenint en compte que iv) hi ha espècies àmpliament distribuides a Europa, com és el cas del cérvol (*Cervus elaphus*).

A Europa, les poblacions de *Culicoides* presents en ecosistemes naturals en proximitat a remugants salvatges eren desconegudes fins el moment. Els resultats demostren que al llarg de la geografia espanyola les zones naturals amb altes densitats de remugants salvatges són zones amb gran diversitat i abundància d'èspècies de *Culicoides*. Les espècies trobades són les mateixes que es poden trobar en explotacions amb remugants domèstics. Les espècies *C. dewulfi*, *C. scoticus*, *C. obsoletus*, C. *imicola* i *C. pulicaris*, reconeguts vectors de la LB i SB, també hi són presents. En cada regió, les espècies vectores presents en contacte amb remugants salvatges coincideix amb les espècies descrites en granges properes amb remugants domèstics (Pla Nacional de Vigilància Entomològica enfront la LB). Les variables que poden afectar la distribució d'aquestes espècies de *Culicoides* vectores, coincideix amb les indicades al primer estudi de la tesi (Capítol I), sent la mitjana de les temperatures màximes del mes més calurós i la taxa de precipitació anual; mentre que el tipus de remugant (domèstic o salvatge) sembla tenir un efecte escàs. La distribució de la principal espècie vectora de la LB en la conca Mediterrània (Mellor *et al.*, 2002; Nolan *et al.*, 2008; Zientara *et al.*, 2009), *C. imicola*, es centra en les quatre àrees mostrejades del centre i sud peninsular coincidint amb les zones més caluroses, i absent en les zones estudiades situades al nord peninsular. Tot el contrari succeeix amb les espècies pertanyents al grup Obsoletus que predominen en zones amb temperatures mitjanes anuals baixes (Purse *et al.*, 2004; Calvete *et al.*, 2008). Les espècies del grup Pulicaris es van capturar en totes les zones mostrejades amb abundàncies mitjanes.

Cal destacar també la major abundància en les zones de remugants salvatges mostrejades de femelles d'espècies de *Culicoides* amb hàbits alimentaris indefinits o ornitofílics. Aquestes espècies són menys abundants en granges tot i utilitzar el mateix sistema de captura, les trampes CDC de llum ultravioleta utilitzades en els Programes de Vigilància Entomològica. Aquest fet és probablement degut a la major diversitat d'hostes dels que alimentar-se i d'ambients on criar.

Una de les vies per aprofundir en el coneixement de la interacció entre vectors i hostes és l'estudi de les preferències d'hoste que tenen els vectors. Aquest és un punt clau per conèixer l'epidemiologia de les diferents malalties que poden transmetre (Mukabana *et al.*, 2002; Lyimo i Ferguson, 2009). Les preferències d'hoste depenen de diferents factors com les característiques intrínseques de cada una de les espècies vectores, així com de

factors ambientals com la disponibilitat dels hostes (Burkot, 1988; Lyimo i Ferguson, 2009). S'han realitzat diversos estudis per caracteritzar les preferències tròfiques dels *Culicoides* però generalment s'han enfocat a detectar espècies amb hàbits mamofílics en zones de remugants domèstics (Mellor *et al*., 2000; Rawlings *et al*., 2003; Bartsch *et al*., 2009; Votypka *et al*., 2009; Garros *et al*.,2011)

Els resultats del Capítol III, mostren que un 80% de les femelles capturades en els ecosistemes naturals ja s'havien alimentat abans (femelles pares i amb sang). La distància a la granja més propera és difícil de sobrevolar per un *Culicoides* en condicions meteorològiques normals (Lilliet *et al*., 1981; Ducheyne *et al*., 2007; Lucientes *et al*., 2008). Això suggereix que la majoria de femelles capturades s'haurien alimentat de fauna salvatge. La posterior identificació genètica de l'origen de la sang detectada a l'abdomen de les femelles de *Culicoides* amb una alimentació de sang recent ens va permetre confirmar aquesta hipòtesi. En els ecosistemes naturals estudiats, les femelles de *Culicoides* semblen tenir preferència pels remugants salvatges en detriment de les aus o altres mamífers. Cal indicar però que la informació no s'ha corregit amb una estimació del cens de mamífers i aus en les zones d'estudi. El cérvol, l'home i la daina van ser els hostes amb un major nombre de deteccions. Així doncs, els resultats avalen que les espècies de *Culicoides* reconegudes com a vectors dels virus de la LB i SB (C*. imicola, C. scoticus, C. obsoletus*, *C. pulicaris i C. punctatus*), podrien actuar com a pont transferint la LB i SB entre els dos tipus de remugants. Entre les espècies exclusivament mamofíliques hi trobem les principals espècies vectores de la LB i SB, però també hi trobem *C. parroti, C. saevus* i *C. yemenensis*. Aquestes darreres espècies en l'actualitat no s'inclouen en el llistat d'espècies d'interès en els Programes de Vigilància Entomològica de la LB a Europa en considerar que no tenen rellevància epidemiològica.

L'espècie *C. parroti* generalment ha estat descrita com espècie amb hàbits alimentaris generalistes (Talavera *et al.,* 2015). Els resultats del Capítol IV ens mostren que en ecosistemes naturals es comportaria com una espècie exclusivament mamofílica. Per altra banda, les captures de *C. parroti* en granja indiquen que és una espècie poc abundant. Tot i això, un estudi realitzat en granja va confirmar que utilitzant un sistema de captura directa aspirant sobre les ovelles, s'obtenien abundàncies elevades de femelles d'aquest espècie a diferència del que succeïa amb les trampes de llum ultraviolada (Gerry *et al.,* 2009). Aquests resultats confirmarien per una banda la proximitat de les femelles de *C.*

*parroti* a les ovelles, confirmant el seus hàbits mamofílics, i per altra banda la infravaloració d'aquesta espècie en les captures realitzades amb trampes DCD de llum ultraviolada. L'explicació plausible de la poca eficiència de les trampes de llum ultraviolada en front la captura de certes espècies de *Culicoides* es creu que podria respondre als hàbits més diürns d'aquestes espècies, que presentarien el seu pic d'activitat en un període del dia en que les trampes CDC-UV encara no són efectives (Carpenter, 2008; Gerry *et al*., 2009; Viennet *et al*., 2011). Altres aspectes fan incrementar el potencial interès de l'espècie *C. parroti*, ja que és una espècie que forma part del subgènere *Monoculicoides*, al que pertanyen les principals espècies vectores de la LB als Estats Units (*C. sonorensis, C. variipennis* i *C. occidentalis*). A més es tracta d'una espècie amb un rang de distribució molt ampli a Europa amb majors abundàncies a la conca Mediterrània (Wilson *et al.*, 2009).

Creiem molt necessari continuar treballant per facilitar la correcta identificació de les diferents espècies de *Culicoides*. La metodologia descrita que integra la morfologia amb tècniques genètiques ha demostrat ser adient per detectar i identificar les espècies de *Culicoides* críptiques presents en una zona. L'esforç s'hauria de centrar en aquells subgèneres amb espècies vectores presents, ja que cada espècie pot tenir un grau diferent de competència vectorial. També aprofundir en el coneixement de les comunitats de *Culioides* en ecosistemes naturals i en especial en contacte amb remugants salvatges, per tal de millorar la comprensió del potencial rol dels remugants salvatges com a reservori d'una malaltia i reintroducció interanual d'aquesta malaltia en una zona. De cara al futur caldria realitzar estudis en les zones interfase de contacte entre remugants domèstics i salvatges (Bartsch *et al*., 2009), utilitzant diferents mètodes de captura. La informació que se'n generi contribuirà a millorar el coneixement de l'epidemiologia de les malalties transmeses per *Culicoides*, i alhora serà d'utilitat per poder realitzar una millor prevenció i control enfront la LB i SB.

**Conclusions**

## Conclusions

L'anàlisi dels resultats obtinguts en aquesta tesi doctoral ens permet concloure que:

1.  L'estudi de la diversitat de *Culicoides* a Catalunya ens ha permès augmentar en 13 el nombre d'espècies conegudes. Destaquen les espècies *C. yemenensis*,nova cita per Europa, *C. coluzzii* i *C. sejfadinei*, noves cites per la Península Ibèrica i *C. pseudopallidus*, nova cita per Espanya.

2.  La distribució de les diferents espècies de *Culciodies* a Catalunya està condicionada per dues variables climàtiques, la mitjana de les temperatures més altes del mes més calurós i la taxa de precipitació anual.

3.  L'estudi morfològic i genètic de *Culicoides* dels grups Pulicaris i Punctatus ha permès la identificació de dues noves espècies críptiques *C. cryptipulicaris* i *C. quasipulicaris*. En conseqüència s'incrementa a 61 el nombre d'espècies que formen el subgènere *Culicoides*.

4.  A Espanya els ecosistemes naturals amb altes densitats de remugants salvatges són zones amb gran diversitat i abundància d'èspècies de *Culicoides. Les espècies* reconegudes com a vectors de la LB i SB hi són presents (*C. dewulfi*, *C. scoticus*, *C. obsoletus*, C*. imicola,C. pulicaris i C. punctatus*).

5.  A nivell regional, la presència de les diferents espècies vectores en ecosistemes naturals amb remugants salvatges coincideix amb la de granges properes de remugants domèstics.

6.  A Espanya , la distribució de les principals espècies de *Culicodies* vectores de la LB i SB (*C. imicola, C. dewulfi, C. obsoletus, C. scoticus* i *C. pulicaris)* està condicionada principalment per dues variables climàtiques, la mitjana de les temperatures més altes del mes més calurós i la taxa de precipitació anual.

7.  Els *Culicoides* capturats en ecosistemes naturals amb presència de remugants salvatges s'alimenten més freqüentment de remugants salvatges, seguit d'altres mamífers i aus.

8. Entre les espècies de *Culicoides* alimentades de remugants salvatges trobem les principals espècies vectores de la LB i SB (*C. obsoletus*, *C. scoticus*, *C. imicola*, *C. pulicaris* i *C. punctatus*), confirmant la hipòtesi que poden actuar com a espècies pont de diferents patògens entre remugants domèstics i salvatges.

**Referències**

# Referències

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