Infection, Genetics and Evolution 11 (2011) 1103–1110

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Adaptation of a species-specific multiplex PCR assay for the identification of blood meal source in *Culicoides* (Ceratopogonidae: Diptera): applications on Palaearctic biting midge species, vectors of Orbiviruses

Claire Garros[a,*], Laëtitia Gardès[a], Xavier Allène[a], Ignace Rakotoarivony[a], Elvina Viennet[a], Sophie Rossi[b], Thomas Balenghien[a]

[a] *Cirad, UMR Contrôle des maladies, F-34398 Montpellier Cedex 5, France*
[b] *Office National de la Chasse et de la Faune Sauvage, Unité Sanitaire de la Faune, Micropolis, la Bérardie, Belle Aureille, 05000 Gap, France*

ARTICLE INFO

Article history:
Received 29 November 2010
Received in revised form 31 March 2011
Accepted 4 April 2011
Available online 12 April 2011

Keywords:
Culicoides
Blood meal analysis
Molecular identification
Trophic behaviour
Bluetongue

ABSTRACT

Culicoides are small biting midges involved worldwide in the transmission of bluetongue and African horse sickness viruses. Feeding behaviours of Palaearctic biting midge species and their spatio-temporal dynamics remain unclear at the specific level. Three multiplex species-specific PCR-based assays were developed and used to identify blood meal source of engorged females of Palaearctic midge species of veterinary interest. Species-specific primers of potential hosts from livestock, domestic animals and wildlife (cattle, goat, sheep, red deer, roe deer, chamois, dog, pig, cat, horse) were designed and multiplexed from the mitochondrial *cytochrome* b gene. The assays also make possible to identify whether multiple blood meals have been taken. The first results from several *Culicoides* populations sampled in France highlight the utility of this valuable diagnostic tool combined with species identification assays, and suggest that most of the *Culicoides* species may have an opportunistic feeding behaviour regarding the host distribution and density. Noteworthy is the peculiar trophic behaviour of *Culicoides chiopterus* showing clear trends to cattle. Information on host preference and feeding behaviours are crucial for a better understanding of vector–host interactions and disease epidemiology.

1. Introduction

The degree of vector–host association is a key predictor of vectorial capacity and transmission of vector-borne pathogens. Understanding host-feeding pattern of vector species populations and their variations in space and time is important because it can contribute to a better knowledge of their respective roles in pathogen transmission, and thus in the design of accurate vector control measures or strategies. Host selection is affected by innate preferences and environmental factors such as host diversity, density and distribution in the insect environment (Lyimo and Ferguson, 2009). Although many studies of host preferences have been conducted for various mosquito or tick vector species (Mukabana et al., 2002; Humair et al., 2007; Lyimo and Ferguson, 2009), it has been a neglected area for biting midges until the spread of bluetongue in Europe.

Culicoides (Diptera: Ceratopogonidae) are small insects responsible for the bluetongue virus transmission to ruminants (Mellor et al., 2009). They are also involved in other *Orbivirus* transmission such as African Horse Sickness virus or Epizootic Haemorrhagic Disease virus. Before its spread through the Mediterranean basin in the late 1990s, bluetongue was considered as an exotic disease restricted to the most southern areas of Europe. The occurrence of this disease over the European territory leads to a dramatic sanitary and economical crisis and shows that some Palaearctic biting midge species are able to transmit the virus intensively; the suspected vectors belonging to the Obsoletus and Pulicaris Groups (Caracappa et al., 2003; Savini et al., 2005; Carpenter et al., 2006; Meiswinkel et al., 2007; Dijkstra et al., 2008).

Most *Culicoides* species are suspected to be mammophilic, biting mostly domestic and wild mammals (Blackwell et al., 1994; Linden et al., 2010). Recent studies in the Palaearctic region showed that *Culicoides obsoletus* was attracted and found blood-fed on sheep or goat (Carpenter et al., 2008; Gerry et al., 2009; Mullens et al., 2010). *Culicoides parroti* has been found feeding on horses and sheep (Mellor and McCaig, 1974; Gerry et al., 2009). *Culicoides impunctatus* is mostly known for its anthropophilic behaviour, while *Culicoides circumscriptus* is considered to be a bird feeder

---

[*] Corresponding author at: UMR 15 Cirad-INRA, Contrôle des maladies animales exotiques et émergentes, Campus International de Baillarguet, TA-A15/A, 34398 Montpellier Cedex 5, France. Tel.: +33 04 67 59 37 01.
E-mail address: claire.garros@cirad.fr (C. Garros).

1567-1348/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2011.04.002

(Blackwell et al., 1994). Some species feed on reptiles and frogs (Votypka et al., 2009).

Despite these studies, many questions remain unanswered regarding feeding patterns of most of biting midge species of veterinary interest. Do species have opportunistic or specialist feeding behaviours? Are there any spatio-temporal variations in feeding patterns? Are there species biting wild ruminants?

Development of PCR-based assays to identify blood meals from sympatric wild and domestic mammals will help answer these questions. Earlier investigations had used immunological assays (Murray, 1970; Braverman et al., 1971, 1977; Tempelis and Nelson, 1971; Braverman and Phelps, 1981; Blackwell et al., 1994) but these tools have several inherent problems (see review in Mukabana et al., 2002). The development of PCR-based technologies provides a more direct approach to identify host species and increases sensitivity and specificity (Mukabana et al., 2002). PCR-based assays using different genetic markers have been developed for mosquitoes targeting potential hosts for malaria or West Nile research purposes (pig, human, goat, dog, and cow; and avian orders respectively) (Ngo and Kramer, 2003; Kent and Norris, 2005). Mitochondrial DNA such as cytochrome b or cytochrome oxidase I (COI) are mostly selected because they exhibit a high level of interspecific polymorphism which helps to design species-specific primers (Hebert et al., 2003). A recent paper proposed a PCR-based method to identify blood meal origins of *Culicoides* but the number of host species available was limited (Bartsch et al., 2009). Moreover, species identification at the specific level was not done; it is difficult thus to draw conclusions on species feeding behaviours. Lassen et al. (2010) and Ninio et al. (2010) studied blood meal origins in Denmark and France respectively, using sequencing followed by comparison of sequence homology through GenBank. Although this method is accurate, it requires time (two main steps: PCR amplification and sequencing) and is limited by the sequences present in GenBank.

The purpose of this study was to develop PCR-based assays to identify blood meal origin from potential livestock, domestic animals and wild ruminants in *Culicoides* females and to use this molecular tool on field-collected engorged females sampled in various localities over France using Onderstepoort black-light traps localized in farms closed to livestock. Garcia-Saenz et al. (2010) demonstrated that female catches in light traps increased linearly with baited animal numbers (sheep in the study), at least for limited host numbers. These results make consistent the assumption that baited light traps collect host-seeking females attracted by the presence of animals and therefore one can hypothesize that engorged females collected by such traps are expected to have feed on the nearest animal, the animal baits.

2. Materials and methods

2.1. DNA extraction, primer design and validation with blood samples

Host primers were selected from Tobe and Linacre (2008) for identifying cattle, sheep, goat, red deer, dog, cat, pig and horse and designed by the authors for chamois and roe deer based on cytochrome b (cytb) polymorphism (Table 2). Primers were designed manually and confirmed using Webprimer (http://www.yeastgenome.org/cgi-bin/web-primer). The location for primer sequences was chosen on the criteria of at least three nucleotide differences between host species and PCR product sizes easily distinguishable by agarose gel electrophoresis (minimum of 20 nucleotides of differences).

EDTA-blotted blood collected in vet clinics from domestic hosts (cattle, goat, sheep, dog, cat, horse) and EDTA-blotted blood or spleen from wild ruminants collected by ONCFS were DNA-extracted using a commercial kit (DNeasy Tissue & Blood, Qiagen, Valencia, CA, USA) and used as positive controls. Abdomens of engorged biting midge females were separated from the body. DNA

Table 1
Characteristics of the six farms where *Culicoides* were collecting from week 12 to 22 in 2009.

Farm code	GPS location and administrative unit	Host diversity and host abundance	Presence of wild ruminants	Trap localization
Farm A	00°38′30″, 45°58′47″ Charente Maritime	140 sheep plus lambs	Wild boars, roe deer	Inside animal shelter, widely open
Farm B	01°44′39″, 43°20′00″ Pyrénées Atlantiques	180 sheep plus lambs	Wild boars, red deer, roe deer	Inside animal shelter, widely open
Farm C	00°46′56″, 43°38′45″ Gers	50 cattle	Wild boars, roe deer	Inside animal shelter, closed building
Farm D	01°39′50″ 48°26′58″ Ile et Vilaine	140 cattle, 6 sheep plus lambs	Wild boars, roe deer	Outside, closed to cattle
Farm E	02°53′13″, 47°05′11″ Cher	200 goats, 75 cattle	Wild boars, red deer, roe deer	Inside animal shelter with little openings

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

Reverse primers		Primer sequence (5′ → 3′)	Length (bp)
	Forward primer UNIV1	GACCAATGATATGAAAAACCATCGTTGT	
Dog *Canis lupus familiaris*	CANIS	CAAGCATACTCCTAGTAAGGATCCG	170
Pig *Sus scrofa*	SUS	TCTGATGTGTAATGTATTGCTAAGAAC	219
	Forward primer UNIV2	TGAGGACAAATATCATTYTGAGGRGC	
Chamois *Rupicapra rupicapra*	RUPI	TGAGGGTAGCCTTGTCTACCGAGAAGCCT	90
Red deer *Cervus elaphus*	CERVUS	AGTAAGTGTACTATAGCGAGTGCTGCG	188
Roe deer *Capreolus capreolus*	CAPREOLUS	TTGTCCGCGTTTGATGGGATTCCTATC	220
Cow *Bos taurus*	BOS	TAAGATGTCCTTAATGGTATAGTAG	287
Goat *Caprus hircus*	CAPRA	TTAGAACAAGAATTAGTAGCATGGCG	313
Sheep *Ovis aries*	OVIS	GGCGTGAATAGTACTAGTAGCATGAGGATGA	336
	Forward primer UNIV3	TTTTTTTTTTTTCGVTCHATYCCHAAYAAACTAGG	
Cat *Felis catus*	FELIS	GATTCATGTTAGGGTTAGGAGATCC	180
Horse *Equus caballus*	EQUUS	TACGTATGGGTGTTCCACTGGC	208