Host feeding patterns of *Culicoides* species (Diptera: Ceratopogonidae) within the Picos de Europa National Park in northern Spain

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Abstract

Blood meal identification can provide information about the natural host-feeding patterns or preferences of *Culicoides* species. Such information could indirectly provide data indicating which reservoirs are significant in associated vector-borne diseases.

We positively identified the host species through DNA sequencing of the *cytochrome b* gene in 144 of the 170 (84.7%) blood meal specimens tested. In the remaining samples, identification of the blood-meal source was unsuccessful, possibly due to the post-ingestion time prior to sampling or the availability of the species-specific *cytochrome b* gene sequences in the database. The majority of identified blood meals were derived from mammalian blood (95.8%), and only six contained chicken blood. We identified five species as mammalian hosts for *Culicoides* spp.: sheep (87.7%), human (6.5%), cattle (3.7%) and Savi's Pine Vole (*Micrototus savii*) (2.1%). The results suggested that large mammals, specifically ruminants, were most frequently fed upon by biting midges (*Culicoides* spp.), but evidence of opportunistic feeding behaviour was also found. Host feeding behaviour of *Culicoides* species may also be influenced by the relative abundance of a particular host species in the area being studied. In this sense, Savi's Pine Vole, a wild species, was found to be a locally relevant host and a putative reservoir for viruses transmitted by species of biting midges belonging to the *Culicoides* genus.

Finally, feeding on multiple potential host species was observed. One midge acquired blood meals from human and chicken hosts, while four other midges fed on two different sheep.

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Introduction

Bluetongue (BT) and African horse sickness (AHS) are two non-contagious, arthropod-borne viral diseases that affect ruminants (both domestic and wild) and horses, respectively. Bluetongue disease is caused by the bluetongue virus (BTV), the prototype of the *Orbivirus* genus within the Reoviridae family, which also includes AHSV (Holmes *et al.*, 1995). Both viruses are transmitted by species of biting midges belonging to the *Culicoides* genus (Diptera: Ceratopogonidae) (Du Toit, 1944; Mellor & Pitzolis, 1979; Mellor *et al.*, 1990) and are maintained naturally through a series of alternative cycles of replication between *Culicoides* vectors and susceptible hosts (Takamatsu *et al.*, 2003).

In the Mediterranean Basin and in southern Europe, the main vector for both viruses is *Culicoides imicola* (Mellor, 1996), although other Palaearctic *Culicoides* species, mainly within the subgenera *Avaritia* and *Culicoides*, contain midges (*C. obsoletus*, *C. scoticus*, *C. dewulfi* and *C. pulicaris*) known or suspected to be BTV vectors (De Liberato *et al.*, 2005; Mehlhorn *et al.*, 2007, 2009; Meiswinkel *et al.*, 2007, 2008; Calvete *et al.*, 2008; Nielsen *et al.*, 2010). In northern Europe and Spain, species of the *Culicoides pulicares* and the *Culicoides obsoletus* groups (*C. obsoletus*, *C. scoticus* and *C. dewulfi)* are involved in bluetongue pathology (Mehlhorn *et al.*, 2009; Nielsen *et al.*, 2010).

Blood meal identification provides information on host-feeding preferences or host-feeding patterns of insects in nature. Such information could indirectly provide data indicating which reservoirs are significant in vector-borne diseases (Lee *et al.*, 2002). Studies involving biting midges (*Culicoides* spp.) suggest that large mammals, specifically ruminants (Bartsch *et al.*, 2009; Gerry *et al.*, 2009; Mullens *et al.*, 2010), are the preferred host, but little is known about the transmission dynamics and the role of wild species. Bartsch *et al.* (2009) determined by PCR that cattle, on selected farms with several species, were the most attractive host for *Culicoides obsoletus* and *Pulicaris* groups even if other large mammals were located in their immediate vicinity. The aim of the present study was to identify blood meals and establish feeding patterns of *Culicoides* spp. in Picos de Europa National Park in northern Spain, where livestock and wildlife coexist. Blood meals were identified by sequencing PCR products of the *cytochrome b* gene from mitochondrial DNA in *Culicoides* species.

Material and methods

Study area

The field work was conducted in the Picos de Europa National Park, 20 km from the northern coast of Spain (centred on 43°15′N, 5°00′W) (fig. 1). The Picos de Europa is a predominantly limestone mountain range with a marked elevation gradient from 200 to 2600 m a.s.l. The climate is extremely wet, particularly in the northern slopes, with precipitation values exceeding 1500 mm year^{-1} in most sites. In the Picos de Europa National Park, extensive livestock rearing has been traditionally practised by small family-based units with an average of 10–12 cows and 50 sheep and/or goats (De Sebastian, 1997). Transhumance is common, with animals moving seasonally from the valley bottoms, where they spend the coldest months of winter, to common mountain pastures in summer, where the livestock are allowed to free-range. Wildlife and livestock coexist in the study area.

Insect samples

Trapping was performed using a 4 W ultraviolet light trap fitted with a suction fan, which is recommended to trap *Culicoides* spp. (Miniature Blacklight Model 1212, John Hock Company, Gainesville, FL, USA). Three traps were placed 1.7 to 2.5 m above ground level near riverbanks and pastures (fig. 1). Trap 1 was located at 1057 m a.s.l. and close to a shepherd house which is occupied in summer. Traps 2 and 3 were located at 842 and 1062 m a.s.l., respectively. All collected insects were transferred into micro tubes, preserved in 70% ethanol and then transported to the laboratory. Tubes were kept frozen (− 20°C) until species identification and DNA extraction. In our work, time between capturing and processing the blood meal analysis were four months. Upon examination, species of *Culicoides* were separated from other insects, with identification to species level based on previously described wing patterns (Rawlings, 1996). In the summer of 2008, 158 fully fed females were randomly selected (table 1) for further analysis. In this paper, the *Culicoides obsoletus* group refers to three putative species: *C. obsoletus*, *C. scoticus* and *C. dewulfi*.

DNA extraction, polymerase chain reaction (PCR) amplification and DNA sequencing

Total DNA was extracted from single midges using a commercial kit (Nucleo Spin Tissue, Mancherey-Nagel, Düren, Germany). Isolated DNA from the midge and blood-meals served as a template for subsequent PCR amplification. All DNA templates were screened with avian and mammalian specific primer pairs for the *cytochrome b* gene (*cytB*) with expected product sizes of 508 and 772 bp, respectively (Molaei *et al.*, 2006). Primers used for specific avian amplification were 5′-GAC TGT GAC AAA ATC CCN TTC CA-3′(forward) and 5′-GGT CTT CAT CTY HGG YTT ACAAGA C-3′(reverse). Mammalian specific PCR primers were 5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′(forward) and 5′-TGT AGT TRT CWG GGT CHC CTA-3′(reverse). Because of sequence homology among classes, these primer sets also amplify reptilian DNA. PCR was performed in a final volume of 25 μl containing 200 ng of total DNA, 10 pmol of each primer, 200 μM dNTPs, 2.5 mM MgCl$_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 0.1 μl of PCR enhancer (wonder-tool, Biotools, Spain) and 1 U *Taq* + 0.5 U *Pfu* polymerases (Biotools, Spain). Forty-five cycles were performed with the following step-cycle program: strand denaturation at 94°C for 45 s, primer annealing at 60°C for avian and 57°C, for mammalian primer pairs for 45 s, and primer extension at 72°C for 40 s. Two PCR amplifications were carried out for avian or mammalian discrimination in each specimen, obtaining a product size of 508 and 772 bp, respectively. Positive PCR products were purified using a commercial kit (Nucleo Spin Extract, Mancherey-Nagel, Düren, Germany) and were sequenced using an ABI Prism 3700 (Applied Biosystems). PCR products were directly sequenced using the primers described above. All amplified products were sequenced from both strands. Sequences were identified using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches in the GenBank database to compare fragments. Only matches with at least 98% were accepted.

Table 1. Blood meal sources from *Culicoides* species. GenBank accession numbers are indicated.

	Ovis aries AY879555	*Bos Taurus* NC_006853	*Homo sapiens* NC_012920	*Gallus gallus* HQ285909	*Microtus savii* AY513824
C. Punctatus	40 (2)		3*	3*	1
C. Obsoletus	53 (2)	4	4	2	2
C. Festivipennis	18		1		
C. Newsteadi	1				
C. Pulicaris	8	1	1	1	
C. Parroti	1				
Total	**121**	**5**	**9**	**6**	**3**

Asterisk shows one midge that has acquired blood meals from humans and chickens. Numbers in brackets indicate number of midges with blood from two different sheep.
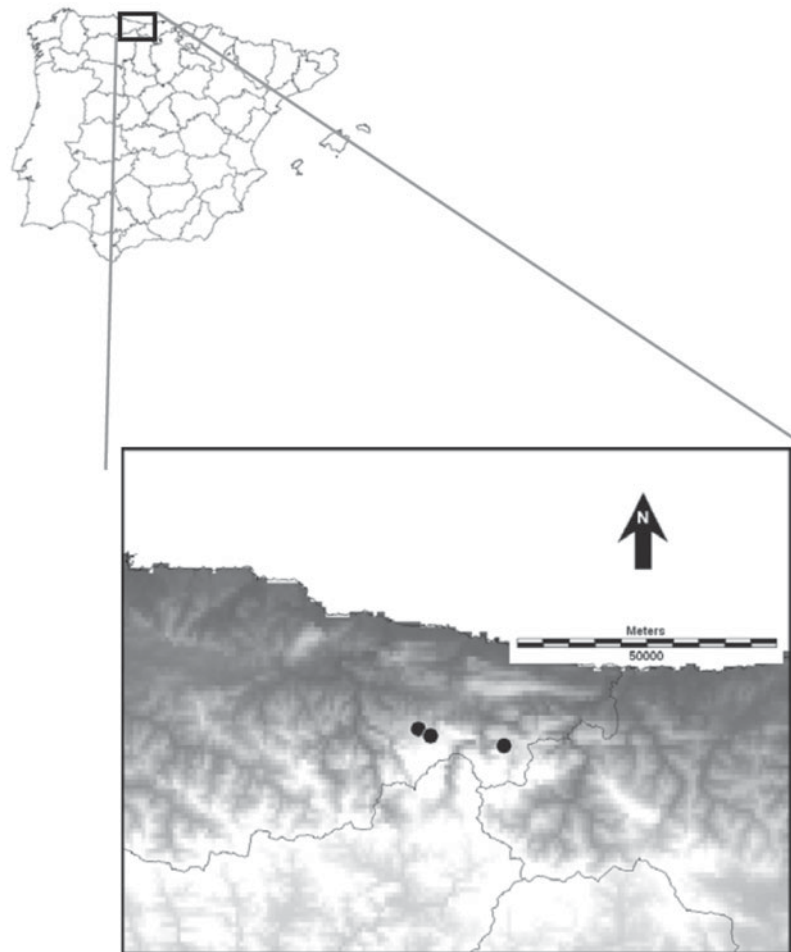

Fig. 1. Location of the study area (northern Spain) and the geographical distribution (black dots) of sampled points. The digital elevation model of the area is shown in grey. Darker and clearer areas represent lower and higher altitudes, respectively.

Results and discussion

We identify the host species by DNA sequencing in 144 of 170 (84.7%) specimens tested (table 1). In the remaining samples, identification of the blood-meal source was unsuccessful, possibly due to the post-ingestion time prior to sampling, time between capturing and processing the blood meal analysis or availability of the species-specific *cytochrome b*

gene sequences in the database. In our study, eight of the 23 unsuccessful attempts at identifying the blood-meal source used mammalian specific primer pairs. The amplified product generated weak bands, and it was not possible to obtain high quality sequences to compare fragments in GenBank, suggesting that host DNA concentrations were low or degraded in the source extractions. Additionally, several studies have demonstrated the important effects that
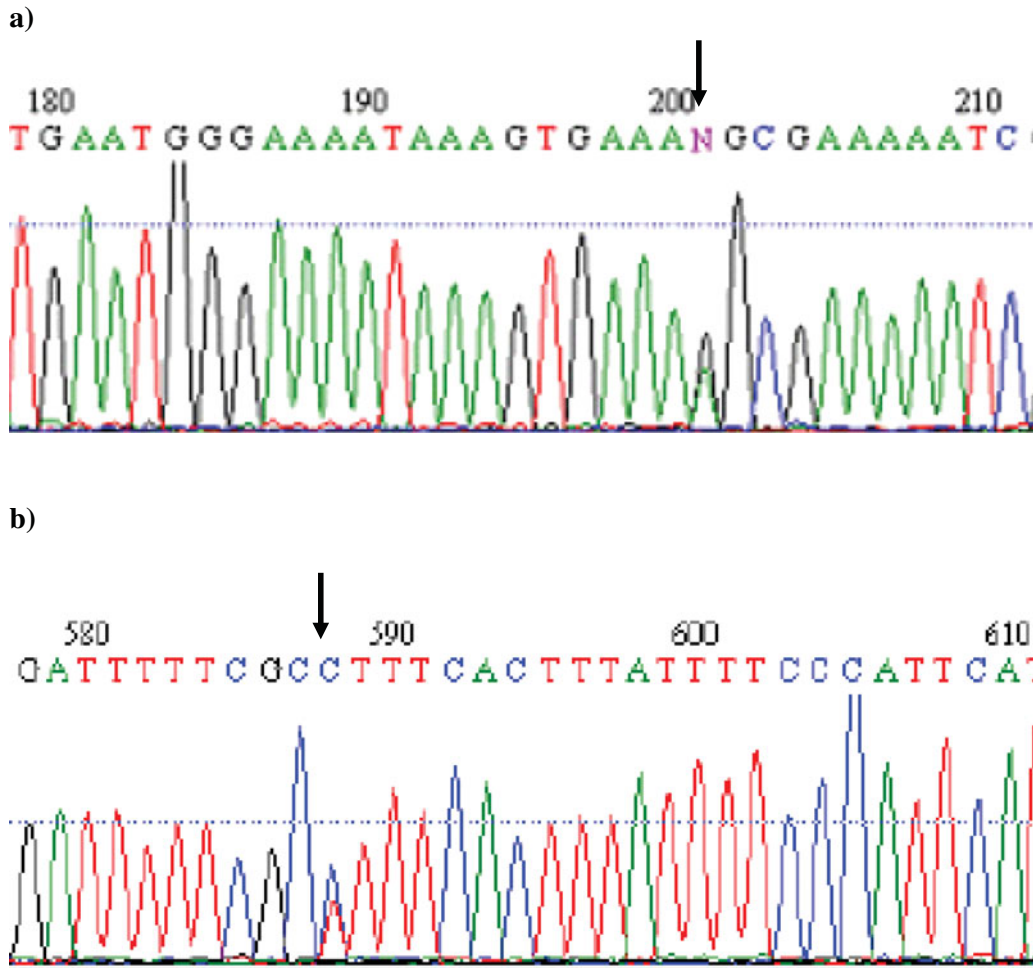
a)

b)

Fig. 2. Sequencing chromatograms obtained by sequencing of the PCR products by the (a) forward and (b) reverse primers of a midge fed on two different sheep. Arrow shows heterozygous position, and then two different haplotypes.

post-ingestion time and the physical conditions under which sacrificed insects are stored can have on the success of detecting blood meal DNA (Mukabana *et al.*, 2002; Oshaghi *et al.*, 2006; Haouas *et al.*, 2007). Oshaghi *et al.* (2006) showed that host DNA extracted from a blood meal up to 33 h post-ingestion acts as an efficient template for PCR amplification. However, meals digested for 36 h or longer required higher DNA concentrations to obtain successful PCR amplification. Haouas *et al.* (2007) found a DNA detection sensitivity varying from 100% to 66% for blood digested from 0–18 h to 24–72 h, respectively, with an overall of 79% positive results in the blood-meal PCR assay. In our study, overall, 84.7% of the blood fed midges showed positive identification. It is also highlighted that the sensitivity depends on both the quantity and degradation of target DNA. *Culicoides* spp. vary in size within and between species, and blood meal volume varies from 0.01 to 0.06 µl (Venter *et al.*, 2005). This blood meal volume is lower than many other insects, such as sand flies (1 µl) (Haouas *et al.*, 2007), tsetse flies (37 µl) (Torr & Hargrove, 1998) or anopheline mosquitoes, which are able to concentrate erythrocytes during the blood feeding process (Briegel & Rezzonico, 1985).

Furthermore, only matches with at least 98% were accepted in our study; three samples did not match exactly the DNA sequences available in the GenBank database. Specifically, sequence matches with 93%, 95% and 91% identity with *Lamprolepis smaragdina* (GenBank accession number AY217803) (*C. obsoletus*), *Tragulus javanicus* (GenBank accession number AY121994) (*C. pulicaris*) and *Francolinus hartlaubi* (GenBank accession number U90639) (*C. festivipennis*), respectively, were found. Because these species of wildlife are not known to be present in Spain, the results indicate that the blood host identity for these midges could be a related species, but we could not conclude which one.

The majority of identified blood meals were derived from mammalian blood (95.8%), and only six contained chicken blood. These blood meals may have come from chickens (*Gallus gallus*; HQ285909) that are part of a conservation programme of an autochthonous chicken breed that is settled in the Picos de Europa National Park. We identify five species as mammalian hosts for *Culicoides* spp. A majority of the blood meals ($n = 121$, 87.7%) were from sheep, followed by human (*Homo sapiens*; GenBank accession number NC_012920) ($n = 9$, 6.5%), cattle (*Bos taurus*; GenBank accession number

NC_006853) (*n*=5, 3.7%), and Savi's Pine Vole (*Micrototus savii*; GenBank accession number AY513824) (*n*=3, 2.1%). Table 1 shows the number of blood meals identified from each of the *Culicoides* spp. sampled in the Picos de Europa National Park in the summer of 2008. This study found that large mammals, specifically ruminants, were the preferred host for biting midges (*Culicoides* spp.), but opportunistic feeding behaviour was also found (human, chicken and Savi's Pine Vole were also identified). Host feeding behaviour of *Culicoides* species may also be influenced by the relative abundance of other possible host species in the study area. In this regard, the wild species, Savi's Pine Vole (*Micrototus savii*), was found to be of relevance as a host and as a putative reservoir for viruses transmitted by species of biting midges belonging to the *Culicoides* genus. Furthermore, the DNA sequencing results from the three blood-meal species showed high identity for three wild species (*Lamprolepis smaragdina*, *Tragalus javanicus* and *Francolinus hartlaubi*), providing additional support for the existence of opportunistic feeding behaviour on non-domestic species. Bartsch *et al.* (2009) determined that cattle located on farms with multiple species were the most attractive host for *C. obsoletus* and *Pulicaris* groups, even if other large mammals were located in the immediate vicinity. Surprisingly, none of the biting midges sampled in that study were found to be positive for sheep's blood. This finding differs from our work and is most likely reflective of the blood meal source being dependent on the attractiveness and the numeric availability of the host. In the Picos de Europa National Park, domestic sheep are much more numerous than cattle. Midges fed on humans and chicken were trapped in the same sample point, close to a shepherd house which is occupied in summer. This was the place where chicken was located.

Other described methods, such as PCR-RFLPs (Maleki-Ravasan *et al.*, 2009; Oshaghi *et al.*, 2009) or multiplex PCR (Haouas *et al.*, 2007) are based on identification of inter specific polymorphisms in the *cytochrome b* gene between species, and the digestion with specific restriction enzymes or the design of specific primers, respectively. In this works, the blood meal origin was identified obtaining different specific species profiles. However, the RLFP or multiplex PCR techniques are based on multiple alignments of the mitochondrial *cytochrome b* gene, and then only species from the multiple alignment can be identify. In our work, the sequencing method used spends more time in the identification but has the advantage to be able to detect any species with an identified sequence deposited in GenBank, including wild species.

Finally, the results showed that a surprising 3.4% of blood-meals were from two different hosts. One midge may have acquired blood meals from human and chicken sources, and then specifics bands of 508 and 772bp for avian and mammalian amplifications were obtained, respectively. Sequence data confirmed the two different origin of the blood. Four other midges fed on two different sheep, as evidenced by the observation of two distinct sheep haplotypes (fig. 2), and confirmed by sequencing of the PCR products by the forward and reverse primers. The *cytochrome b* gene is located in the mitochondrial DNA, which is haploid and maternally inherited, and heterozygous mitochondrial DNA is very rare and not reported in sheep. However, several studies have demonstrated the presence of leaked paternal mitochondrial DNA (biparental transmission of mitochondrial DNA), particularly in interspecific hybrids between closely related species in several genera, such as *Mytilus* (Zouros *et al.*, 1994; Rawson *et al.*, 1996), *Drosophila* (Kondo *et al.*, 1990) and *Mus* (Gyllensten *et al.*, 1991; Kaneda *et al.*, 1995). The observation of a heterozygous nucleotide in the sequencing chromatogram could indicate two haplotypes and then multiple feeding. Homology searches of the two haplotypes showed 100% identity with sheep. Furthermore, this substitution was conservative, and no amino acid change was observed in the protein sequence between the two haplotypes. Multiple feedings on potential hosts within the same gonotrophic cycle may enhance amplification and transmission of viruses carried by these vectors. However, multiple feeding may or may not favour disease transmission, depending on whether the vector feeds on potential or dead-end hosts (Arunachalam *et al.*, 2005).

In conclusion, these results reveal that biting midges of *Culicoides* spp. can feed on a variety of vertebrate hosts including mammals, birds and wild species, with a preference for ruminants. Evidence for opportunistic feeding behaviour on wild species was found along with feeding on multiple potential hosts.

Acknowledgements

References

Arunachalam, N., Samuel, P.P., Hiriyan, J., Rajendran, R. & Dash, A.P. (2005) Short report: Observations on the multiple feeding behavior of Culex tritaeniorhynchus (Diptera: culicidae), the vector of Japanese encephalitis in Kerala in southern India. *American Journal of Tropical Medicine and Higiene* **72**(2), 198–200.

Bartsch, S., Bauer, B., Wiemann, A., Clausen, P.H. & Steuber, S. (2009) Feeding patterns of biting midges of the *Culicoides obsoletus* and *Culicoides pulicaris* groups on selected farms in Brandenburg, Germany. *Parasitology Research* **105**(2), 373–380.

Briegel, H. & Rezzonico, L. (1985) Concentration of host blood protein during feeding by anopheline mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology* **22**, 612–618.

Calvete, C., Calvo, J.H., Calavia, R., Miranda, M.A., Borrás, D., Estrada, R., Lucientes, J., Mañuz, B. & Romero, L. (2008) *Culicoides* species and transmission of bluetongue virus in Spain. *Veterinary Record* **162**, 255.

De Liberato, C., Scavia, G., Lorenzetti, R., Scaramozzino, P., Amaddeo, D., Cardeti, G., Scicluna, M., Ferrari, G. & Autorino, G.L. (2005) Identification of *Culicoides obsoletus* (Diptera: Ceratopogonidae) as a vector of bluetongue virus in central Italy. *The Veterinary Record* **156**, 301–304.

De Sebastián, J. (1997). Los aprovechamientos ganaderos en los Picos de Europa. pp. 63–76 in B. Palacios, & L. Llaneza (Eds) *Primer Simposio sobre el Lobo en los Picos de Europa*. Oviedo, Spain, Sociedad Española para la Conservación y Estudio de los Mamíferos (SECEM) – Grupo Lobo.

Du Toit, R.M. (1944) The transmission of blue-tongue and horsesickness by *Culicoides*. *Onderstepoort Journal of Veterinary Science and Animal Industry* **19**, 7–16.

Gerry, A.C., Sarto i Monteys, V., Moreno Vidal, J.O., Francino, O. & Mullens, B.A. (2009) Biting rates of *Culicoides* midges (Diptera: Ceratopogonidae) on sheep in northeastern Spain in relation to midge capture using UV light and carbon dioxide-baited traps. *Journal of Medical Entomology* **46**(3), 615–624.

Gyllensten, U., Wharton, D., Josefsson, A. & Wilson, A.C. (1991) Paternal inheritance of mitochondrial DNA in mice. *Nature* **352**(6332), 255–257.

Haouas, N., Pesson, B., Boudabous, R., Dedet, J.P., Babba, H. & Ravel, C. (2007) Development of a molecular tool for the identification of Leishmania reservoir hosts by blood meal analysis in the insect vectors. *American Journal of Tropical Medicine and Hygiene* **77**, 1054–1059.

Holmes, I.H., Boccardo, G., Estes, M.K. & Furuichi, M.K. (1995) Family *Reoviridae. In* Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A. & Summers, M.D. (*Eds*) *Virus Taxonomy: Classification and Nomeclature of Viruses.* Sixth report of the International Committee on Taxonomy of Viruses. New York, USA, Springer, *Archives of Virology* (*Supplement*) **10**, 208–239.

Kaneda, H., Hayashi, J., Takahama, S., Taya, C., Lindahl, K.F. & Yonekawa, H. (1995) Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **92**(10), 4542–4546.

Kondo, R., Satta, Y., Matsuura, E.T., Ishiwa, H., Takahata, N. & Chigusa, S.I. (1990) Incomplete maternal transmission of mitochondrial DNA in Drosophila. *Genetics* **126**(3), 657–663.

Lee, J.H., Hassan, H., Hill, G., Cupp, E.W., Higazi, T.B., Mitchell, C.J., Godsey, Jr, M.S. & Unnasch, T.R. (2002) Identification of mosquito avian-derived blood meals by polymerase chain reaction-heteroduplex analysis. *American Journal of Tropical Medicine and Hygiene* **66**(5), 599–604.

Maleki-Ravasan, N., Oshaghi, M.A., Javadian, E., Rassi, Y., Sadraei, J. & Mohtarami, F. (2009) Blood meal identification in field-captured sand flies: Comparison of PCR-RFLP and ELISA assays. *Iranian Journal of Arthropod-Borne Disease* **3**(1), 8–18.

Mehlhorn, H., Walldorf, V., Klimpel, S., Jahn, B., Jaeger, F., Eschweiler, J., Hoffmann, B. & Beer, M. (2007) First occurrence of *Culicoides obsoletus*-transmitted bluetongue virus epidemic in central Europe. *Parasitology Research* **101**, 219–228.

Mehlhorn, H., Walldorf, V., Klimpel, S., Schaub, G., Kiel, E., Focke, R., Liebisch, G., Liebisch, A., Werner, D., Bauer, C., Clausen, H., Bauer, B., Geier, M., Hörbrand, T., Bätza, H.J., Conraths, F.J., Hoffmann, B. & Beer, M. (2009) Bluetongue disease in Germany (2007–2008): monitoring of entomological aspects. *Parasitology Research* **105**(2), 313–319.

Meiswinkel, R., van Rijn, P., Leijs, P. & Goffredo, M. (2007) Potential new *Culicoides* vector of bluetongue virus in northern Europe. *Veterinary Record* **161**, 564–565.

Meiswinkel, R., Baldet, T., de Deken, R., Takken, W., Delecolle, J.C. & Mellor, P.S. (2008) The 2006 outbreak of bluetongue in northern Europe – the entomological perspective. *Preventive Veterinary Medicine* **87**(1–2), 55–63.

Mellor, P.S. (1996) *Culicoides*: vectors, climate change and disease risk. *Veterinary Bulletin* **66**, 301–306.

Mellor, P.S. & Pitzolis, G. (1979) Observations on breeding sites and light trap collections of *Culicoides* during an outbreak of bluetongue in Cyprus. *Bulletin of Entomological Research* **69**, 229–234.

Mellor, P.S., Boned, J., Hamblin, C. & Graham, S. (1990) Isolations of African horse sickness virus from vector insects made during the 1988 epizootic in Spain. *Epidemiology and Infection* **105**, 447–454.

Molaei, G., Oliver, J., Andreadis, T.G., Armstrong, P.M. & Howard, J.J. (2006) Molecular identification of blood-meal sources in *Culiseta melanura* and *Culiseta morsitans* from an endemic focus of eastern equine encephalitis virus in New York. *American Journal of Tropical Medicine and Hygiene* **75**(6), 1140–1147.

Mukabana, W.R., Takken, W., Seda, P., Killeen, G.F., Hawley, W.A. & Knols, B.G. (2002) Extent of digestion affects the success of amplifying human DNA from blood meals of *Anopheles gambiae* (Diptera: Culicidae). *Bulletin of Entomological Research* **92**(3), 233–239.

Mullens, B.A., Gerry, A.C., Monteys, V.S., Pinna, M. & González, A. (2010) Field studies on *Culicoides* (Diptera: Ceratopogonidae) activity and response to deltamethrin applications to sheep in northeastern Spain. *Journal of Medical Entomology* **47**(1), 106–110.

Nielsen, S.A., Nielsen, B.O. & Chirico, J. (2010) Monitoring of biting midges (Diptera: Ceratopogonidae: *Culicoides* Latreille) on farms in Sweden during the emergence of the 2008 epidemic of bluetongue. *Parasitology Research* **106**(5), 1197–1203.

Oshaghi, M.A., Chavshin, A.R., Vatandoost, H., Yaaghoobi, F., Mohtarami, F. & Noorjah, N. (2006) Effects of post-ingestion and physical conditions on PCR amplification of host blood meal DNA in mosquitoes. *Experimental Parasitology* **112**(4), 232–236.

Oshaghi, M.A., Maleki Ravasan, N., Hide, M., Javadian, E., Rassi, Y., Sadraei, J., Mohebali, M., Sedaghat, M.M., Hajjaran, H., Zarei, Z. & Mohtarami, F. (2009) Vector incrimination of sand flies in the most important visceral leishmaniasis focus in Iran. *American Journal of Tropical Medicine and Hygiene* **81**, 572–577.

Rawlings, P. (1996) A key, based on wing patterns of biting midges (genus *Culicoides* Latreille – Diptera: Ceratopogonidae) in the Iberian Peninsula, for use in epidemiological studies. *Graellsia* **52**, 57–71.

Rawson, P.D., Secor, C.L. & Hilbish, T.J. (1996) The effects of natural hybridization on the regulation of doubly uniparental mtDNA inheritance in blue mussels (*Mytilus* spp.). *Genetics* **144**(1), 241–248.

Takamatsu, H., Mellor, P.S., Mertens, P.P.C., Kirkham, P.A., Burroughs, J.N. & Parkhouse, R.M.E. (2003) A possible overwintering mechanism for bluetongue virus in the absence of the insect vector. *Journal of general Virology* **84**, 227–235.

Torr, S.J. & Hargrove, J.W. (1998) Factors affecting the landing and feeding responses of the tsetse fly *Glossina pallidipes* to a stationary ox. *Medical and Veterinary Entomology* **12**, 196–207.

Venter, G.J., Paweska, J.T., Lunt, H., Mellor, P.S. & Carpenter, S. (2005) An alternative method of blood-feeding *Culicoides imicola* and other haematophagous *Culicoides* species for vector competence studies. *Veterinary Parasitology* **131**(3–4), 331–335.

Zouros, E., Oberhauser Ball, A., Saavedra, C. & Freeman, K.R. (1994) An unusual type of mitochondrial DNA inheritance in the blue mussel Mytilus. *Proceedings of the National Academy of Sciences of the United States of America* **91**(16), 7463–7467.