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RESEARCH ARTICLE

Host Preferences of Vector *Culicoides* (Diptera, Ceratopogonidae, *Culicoides* Latreille) Species in Türkiye

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Abstract: Determination of host preferences of blood-feeding arthropods is an important criterion for how ectoparasite-host interactions, host selection, and feeding behaviour affects pathogen transmission. This study aimed to examine the host's blood from engorged *Culicoides* species collected from cattle farms in Turkey to shed light on their host preferences. For this purpose, we investigated the blood of 5 different hosts (cattle, sheep, dog, horse, and human) in 7 *Culicoides* species/species complexes by multiplex-PCR analysis, considering the domestic animals on the farms. Engorged *Culicoides* specimens were collected from places in Turkey with different geographical and ecological characteristics. A total of 1225 female *Culicoides* belonging to 7 different species/species complexes namely *C. obsoletus* complex (*C. obsoletus* and *C. scoticus*) (n:450), *C. schultzei* complex (n:234), *C. imicola* (n:208), *C. punctatus* (n:162), *C. newsteadi* (n:144), *C. lupicaris* (n:24), and *C. pulicaris* (n:3) were analysed for host blood identification. Abdomens of the engorged midges were separated from their body and pooled according to the date of collection and species. A total of 69 pools consisting of 1-28 specimens were analysed by multiplex-PCR and only cattle blood was detected in all pools. This study presents the first data on the identification of host preference of some *Culicoides* species in Turkey.

Keywords: *Blood meal preferences, Culicoides, multiplex-PCR, Türkiye.*

Türkiye'de Vektör *Culicoides* (Diptera, Ceratopogonidae, *Culicoides* Latreille) Türlerinin Konak Tercihlerinin Belirlenmesi

Öz: Kanla beslenen artropodların konak tercihlerinin tanımlanması, ektoparazit-konak etkileşimleri, eklembacaklıların konak seçimi ve beslenme davranışının patojen bulaşmasını nasıl etkilediği konularında önemli bir kriterdir. Bu çalışmanın amacı, Türkiye'deki sığır çiftliklerinden toplanan doymuş *Culicoides* türlerinden konakçı kanını inceleyerek konak tercihlerine ışık tutmaktır. Bu amaçla çiftliklerde bulunan evcil hayvanlar göz önünde bulundurularak 7 *Culicoides* tür/tür kompleksinde 5 faklı konağın (sığır, koyun, köpek, at ve insan) kanını multiplex-PCR testi ile araştırdık. Doymuş *Culicoides* örnekleri Türkiye'de farklı coğrafi ve ekolojik özelliklere sahip yerlerden toplanmıştır. *Culicoides obsoletus* complex (including *C. obsoletus* and *C. scoticus*) (n:450), *C. schultzei* complex (n:234), *C. imicola* (n:208), *C. punctatus* (n:162), *C. newsteadi* (n:144), *C. lupicaris* (n:24) ve *C. pulicaris* (n:3) olarak belirlenen 7 farklı tür/tür kompleksine ait toplam 1225 adet *Culicoides* konak kanı tespiti için analiz edildi. Doymuş dişilere ait abdomenler gövdelerinden ayrılarak toplanma tarihlerine ve türlerine göre havuzlar oluşturuldu. Toplam 1-28 sinekten oluşan 69 havuz multiplex-PCR ile analiz edildi ve tüm örneklerde sadece sığır kanı tespit edildi. Çalışmamız Türkiye'de *Culicoides* türlerinin konak tercihinin belirlenmesi konusunda ilk verileri sunmaktadır.

Anahtar sözcükler: *Kan emme tercihi, Culicoides, multiplex-PCR, Türkiye.*

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Introduction

Culicoides (Diptera: Ceratopogonidae) are among the smallest hematophagous flies in the world, measuring 1-3 mm in size. The genus is of great interest for its role as biological vector of pathogens of medical and veterinary importance. In addition to several nematode and protozoan species, more than 50 arboviruses have been isolated from *Culicoides* species [1-3]. Their role in transmitting diseases such as bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV), African horse sickness virus (AHSV), Akabane virus, and Schmallenberg virus which causes economic losses in the livestock industry is the main reason for large scale investigation of this genus [4,5]. They are found on nearly all major landmasses from sea level to 4000 m [3]. A total number of 1347 species have been described so far [6]; 72 species have been reported from Turkey [7,8].

Culicoides species can cause severe itchy skin reactions in humans and animals, forcing the hosts to relocate or to flee indoors [1]. Their ability to transmit the Oropouche virus (OROV), the etiological agent of the febrile illness Oropouche fever, among humans has been the most important role of *Culicoides* species in public health to date [3]. A wide variety of insights can be obtained by examining the blood on which arthropods feed, such as the evolution of host specificity between vertebrates and their ectoparasites, ectoparasite-host interactions, the ecology of infectious diseases, how arthropod host selection and feeding behaviour affect pathogen transmission, and the economic and wellness effects of ectoparasite infestation on domestic animals and wildlife [9].

The vast majority of *Culicoides* species feed on mammals and birds; however, reptiles and amphibians are also in the host spectrum of these flies [1,10-12]. Although feeding frequency varies by species and environmental conditions, host availability plays an important role in the feeding behaviour of biting midges in general. Most of the *Culicoides* species are mammalophilic and ornithophilic, although some species are known to feed on reptiles and frogs [1,13-16]. As a result of the molecular identification of vertebrate hosts of *Culicoides* species in Europe, 45 different host species, including 33 bird species and 12 mammal species, were identified [17].

While serological methods were used to determine host preferences in the past [18,19], these have now been replaced by molecular methods. Advances in polymerase chain reaction (PCR)-based assays and molecular techniques for blood-meal analysis using direct sequencing of the cytochrome b (*cytb*) gene allow for species-level identification of hosts with a much higher degree of accuracy than has been achieved with previous serological assays [9-12,20-26]. This study aimed to identify the host's blood in engorged *Culicoides* species collected from cattle farms with other domesticated animals to understand their feeding preferences. This is the first study in Turkey to analyse the host's blood based on a segment of the mitochondrial gene, *cytb*, in *Culicoides* species.

Material and Methods

Study Area

The stations where *Culicoides* specimens were collected were selected from places in Turkey with different geographical and ecological characteristics *(Table 1)*.

Collection of *Culicoides* Specimens

In this study, Onderstepoort Veterinary Institute (OVI) type light traps working with 220V, 8 Watt black fluorescent light and downward fan with a 4 mm net around to prevent the entry of large arthropods were used. Light traps were placed approximately 1.5-2 m above ground out of animals' reach and operated one hour before sunset until one hour after sunrise in cattle farms, where there were at least 20 cattle. There were few sheep, dogs, and a few horses in and around these farms. The samples were obtained from 24 cattle farms in 20 provinces by running traps once a month for 14 months between April-2019 and October-2020 *(Table 1)*. Collected *Culicoides* specimens were morphologically identified under the stereomicroscope using the identification key for Palaearctic *Culicoides* species [27]. *Culicoides schultzei* complex consists of several species *(C. oxystoma*, *C. schultzei*, *C. subschultzei*, *C. kingi*, *C. rhizophorensis*, *C. enderleini*, *C. nevilli*, and *C. neoschultzei)* [28]. Since there is no detailed identification key on the complex, specimens were identified as *C. schultzei* complex. In this study, the abdomens of engorged fully females were separated and pooled according to their species and date of collection, DNA extraction was performed and the DNA samples were stored at -20°C until use.

DNA Extraction and Validation with Blood Samples

Blood of from domestic hosts (cattle, sheep, dog, and horse) was obtained from the animal hospital of Kafkas University Faculty of Veterinary Medicine and human blood obtained from the University Research Hospital. DNA extraction was conducted from each blood sample by using QIAmp DNA Mini Kit (Qiagen, GmbH, Hilden, Germany) in order to obtain positive controls in the PCR assay. These hosts were chosen for the assay because they were potential targets where traps were placed. Abdomens of engorged *Culicoides* females were separated from their bodies and pooled (1-28 per tube) in separate tubes according to date and species. Each pool was digested in 200 mL lysis buffer in tubes with steel balls and extracted according to the kit procedure (Analytik Jena AG, AJ Innuscreen GmbH, Berlin, Germany).

Table 1. *Summary of locations where blood-fed Culicoides were collected*

Province	District/Village	Species	Latitude	Longitude	Altitude (m)	Type of Animals Around the Trap	Blood-Fed Midges, n	Trap Localisations
Artvin	Ardanuç	*C. newsteadi*	41.114820	42.066060	489	Cattle, dog	40	Indoor
		C. obsoletus complex					217	
		C. punctatus					121	
		C. lupicaris					23	
Ankara	Çubuk	*C. newsteadi*	40.118740	32.944540	956	Cattle, sheep, dog	2	Outdoor
Edirne	Merkez/ Budakdoğanca	*C. newsteadi*	41.760760	26.340980	116	Cattle, dog, horse	71	Outdoor
		C. obsoletus complex					5	
		C. punctatus					13	
		C. pulicaris					3	
Edirne	Merkez/İskender	*C. newsteadi*	41.630751	26.669324	93	Cattle, dog, horse	21	Indoor
		C. punctatus					4	
Antalya	Kaş	*C. newsteadi*	36.339800	29.327030	11	Cattle, dog	4	Outdoor
Erzincan	Merkez/Çatalören	*C. newsteadi*	39.664770	39.518180	1191	Cattle, dog	6	Outdoor
Trabzon	Sürmene	*C. obsoletus* complex	40.891111	40.056944	75	Cattle, dog	32	Indoor
Rize	Fındıklı	*C. obsoletus* complex	41.253880	41.156770	30	Cattle, dog	137	Indoor
Mersin	Anamur	*C. obsoletus* complex	36.133480	32.859470	54	Cattle, sheep, dog	8	Indoor
		C. imicola					84	
Mersin	Silifke	*C. schultzei* complex	36.335278	34.000833	0,3	Cattle, dog	16	Outdoor
Samsun	Atakum	*C. obsoletus* complex	41.433580	36.084770	204	Cattle, dog	15	Indoor
		C. lupicaris					1	
Bursa	İznik	*C. obsoletus* complex	40.541110	29.834200	850	Cattle, sheep, dog	2	Indoor
Kastamonu	Araç	*C. obsoletus* complex	41.218780	33.381380	852	Cattle, sheep, dog	23	Indoor
Erzurum	Şenkaya	*C. obsoletus* complex	40.640000	42.338889	1247	Cattle, dog	2	Indoor
Erzurum	Şenkaya/Aydoğdu	*C. punctatus*	40.701246	42.471634	1631	Cattle, dog	20	Indoor
Kırklareli	Vize	*C. obsoletus* complex	41.706903	27.704541	430	Cattle, sheep, dog	2	Indoor
Balıkesir	Karasi	*C. obsoletus* complex	39.894986	27.843145	280	Cattle, sheep, dog	3	Indoor
Giresun	Merkez	*C. obsoletus* complex	40.910409	38.313453	35	Cattle, dog	4	Indoor
Burdur	Merkez	*C. punctatus*	37.633690	30.106700	875	Cattle, dog	4	Outdoor
Hatay	Arsuz	*C. imicola*	36.406389	35.891111	7	Cattle, dog	49	Outdoor
		C. schultzei complex					101	
Diyarbakır	Sur	*C. imicola*	37.961110	40.428700	641	Cattle, dog	5	Indoor
Diyarbakır	Bismil	*C. schultzei* complex	37.826090	40.615240	554	Cattle, dog	52	Outdoor
Gaziantep	Nurdağı	*C. schultzei* complex	37.190680	36.843299	506	Cattle, sheep, dog	50	Outdoor
		C. imicola					70	
Adana	Kozan	*C. schultzei* complex	37.363219	35.716955	90	Cattle, sheep, dog	15	Outdoor

Species-specific Multiplex PCR Assay

The primer sets used in this study are given in *Table 2*. Separate reactions were prepared for each primer set *(Fig. 1)*. Multiplex-PCR was performed with UNREV1025 in a total volume of 25 µL using 2.5 µL of 10X PCR buffer, 2.5 µL of 2 mM $MgCl_2$, 1 µL of 10 mM dNTP mix, 1 µL of each primer (10 pmol), 0.125 µL Taq polymerase and 4 µl template DNA. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sEC, 57°C for 1min, 72°C for 1min, and final elongation at 72°C for 5min. The PCR products were loaded onto 1.5% agarose gel stained with SYBR® Safe DNA (Thermo Fisher Scientific, Invitrogen) and visualized under UV light.

Table 2. *Primer set used for the identification of blood-meal in Culicoides abdomens*			
Primer Name	**Primer Sequence (5'→3')**	**Length (bp)**	**Origin**
UNREV1025	GGTTGTCCTCCAATTCATGTTA		Kent and Norris [29]
Human741	GGCTTACTTCTCTTCATTCTCTCCT	334	Kent and Norris [29]
Cow121	CATCGGCACAAATTTAGTCG	561	Kent and Norris [29]
Horse	CCCTACATCGGTACTACCC	500	Pitzer at al.[30]
Forward primer UNIV1	GACCAATGATATGAAAAACCATCGTTGT		Garros at al.[21]
Dog, *Canis lupus* familiaris	CAAGCATACTCCTAGTAAGGATCCG	170	Garros at al.[21]
Forward primer UNIV2	TGAGGACAAATATCATTYTGAGGRGC		Garros at al.[21]
Sheep *Ovis aries*	GGCGTGAATAGTACTAGTAGCATGAGGATGA	336	Garros at al.[21]
Cow, *Bos taurus*	TAAGATGTCCTTAATGGTATAGTAG	287	Garros at al.[21]
Forward primer UNIV3	TTTTTTTTTTTTCGVTCHATYCCHAAYAAACTAGG		Garros at al.[21]
Horse, *Equus caballus*	TACGTATGGGTGTTCCACTGGC	208	Garros at al.[21]

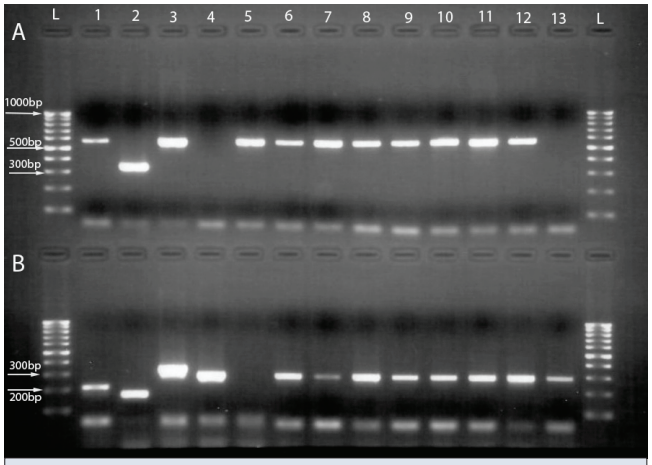

Fig 1. Species-specific PCR amplifications. **A-** Unrev1025 primer set used; L: 100 bp molecular DNA ladder, 1. blood DNA template from a horse, 2. blood DNA template from human, 3. blood DNA template from cattle, 4. Negative control, 5-12. Samples. 13. Negative control **B**-UNIV1, UNIV2 and UNIV3 primer sets used; L: 100 bp molecular DNA ladder, 1. blood DNA template from a horse, 2. blood DNA template from a dog, 3. blood DNA template from sheep, 4. blood DNA template from cattle, 5. Negative control, 6-13. Samples

Results

In this study, 1225 engorged *Culicoides* females were identified from 24 traps in 20 provinces, which belong to 7 different *Culicoides* species/species complexes: *C. obsoletus* complex *(C. obsoletus* and *C. scoticus)* (n:450), *C. schultzei* complex (n:234), *C. imicola* (n:208), *C. punctatus* (n:162), *C. newsteadi* (n:144), *C. lupicaris* (n:24) and *C. pulicaris* (n:3). Engorged females of *C. obsoletus* complex were collected from 12 different traps, *C. newsteadi* from 5 different traps, *C. punctatus* from 4 different traps, *C. lupicaris* from 2 different traps, *C. pulicaris* from only 1 trap, *C. imicola* from 4 different traps and *C. schultzei* complex from 5 different traps *(Table 1)*. Engorged abdomens were separated and pooled according to the date of collection and species. A total of 69 pools consisting of 1-28 specimens were analysed. DNA from human, sheep, cattle, dog, and horse blood were amplified with species-specific *cytb* primers *(Fig. 1)*. As a result of the molecular analysis of 69 pools, all 7 *Culicoides* species/ species complexes were found to be fed only from cattle *(Table 3)*.

Table 3. *Blood-meal identifications by multiplex-PCR of engorged Culicoides.*					
***Culicoides* Species**	**Cow *(Bos taurus)***	**Sheep *(Ovis aries)***	**Horse *(Equus caballus)***	**Human *(Homo sapiens)***	**Dog (Canis lupus)**
C. newsteadi	+	-	-	-	-
C. obsoletus complex (*C. obsoletus*/*C. scoticus*)	+	-	-	-	-
C. punctatus	+	-	-	-	-
C. lupicaris	+	-	-	-	-
C. pulicaris	+	-	-	-	-
C. imicola	+	-	-	-	-
C. schultzei complex	+	-	-	-	-

Discussion

Molecular techniques developed to identify vertebral hosts that blood-feeding arthropods feed on, allow the host to be determined at the species level [9,17]. In the identification of host preferences of engorged *Culicoides* species, species-specific primers based on the *cytb* that amplify the known host DNA [20,21] and general primers that amplify the conserved genetic regions of all vertebrate species are used [10,22,23,25,26]. In this study, we performed a multiplex-PCR test using specific primers based on *cytb* polymorphism and determined the host blood (bovine, sheep, dog, equine and human) in 7 *Culicoides* species/species complexes. Multiplex-PCR test is a fast, cost-effective, and efficient method that can be routinely applied in the laboratory, where large numbers of samples are tested simultaneously. It is widely used to identify the host preference of various blood-feeding arthropods [17,21,30]. However, it requires preliminary selection to identify primer sets for targeted hosts, which may cause a significant limitation in samples from undisturbed habitats with a wider host range [17,21]. We did not encounter the stated adverse effects as there were a limited number of target hosts (cattle, sheep, dog, horse, and human) in and around the farms where the light traps were placed. In this study, as a result of multiplex-PCR, 7 *Culicoides* species/species complexes *(C. obsoletus* complex, *C. schultzei* complex, *C. imicola*, *C. punctatus*, *C. newsteadi*, *C. lupicaris*, and *C. pulicaris)* were found to feed on cattle but not on other hosts. This may be due to the small number of other hosts in the farms, the higher CO2 emission, the larger mass of cattle and the greater number of cattle in the farms [20]. It has been stated that the host selection of *Culicoides* species may be limited by the presence of hosts in their environment [15,17]. Lassen et al.[12] found 74% cattle blood from the collected samples of *Culicoides* species in their study, and they stated that the result was due to placing the traps close to cattle. In our study, all the traps were placed in cattle farms. As a result, cattle appear to be more attractive hosts for *Culicoides* due to their greater number, less mobility than other animals, and limited defensive movements. In a study on the host preference of *Culicoides* between cattle, horse, and sheep, it was found that *Culicoides* preferred cattle more in an area where cattle and sheep coexist, while more species and individuals were collected from horses [31]. It has been reported that cattle not only attract *Culicoides* but are also important hosts of for these species [13]. Studies on host preferences are also performed by direct aspiration of species from hosts, sweep nets or drop nets. Comparison of host preference between cattle and sheep with these methods showed that cattle are far more attractive hosts for *Culicoides* species in general [13,32]. The structure of the farms (indoor or outdoor), seasonal behavioural changes, and the number of hosts are important for host selection of *Culicoides* species [12].

Culicoides imicola is one of the most important vector species among the species analysed for host identification in this study [3]. *C. imicola* is an important vector for bluetongue virus (BTV) and is distributed in the south and southeast regions of Türkiye [33]. There are few number of researches on the host preference of this species. Slama et al.[34] identified host preferences of *C. imicola* in 96 engorged females and found that they feed on cattle, humans, sheep, goats, and dogs. Martínez-de la Puente et al.[23] found in their study that *C. imicola* feed on 6 mammal species. In a study carried out in South Africa, it has been detected that *C. imicola* and *C. subschultzei* fed on human, zebra, and kudu blood [35]. Both species *(C. imicola* and *C. schultzei* complex) were found to feed on blood from cattle in this study.

Newly engorged female specimens are important in identifying the host source with high accuracy. However, it is known that it is quite difficult to collect these newly engorged females. It represents only a small portion of the total individuals captured [17]. For this reason, it is stated that host preference identification can also be made with *Culicoides* at different stages of digestion [36]. In our study, 1225 new engorged *Culicoides* specimens were analysed. In future studies, choosing samples from both newly engorged specimens and specimens at different stages of digestion (partially or completely) will expand the number of samples for host identification studies.

Studies carried out in Turkey so far have mainly focused on the *Culicoides* species fauna [7,8,37,38]. Our study presents the first data on the identification of host preference of *Culicoides* species in Turkey. In this study, we found that 7 *Culicoides* species/species complexes (*C. obsoletus* complex, *C. schultzei* complex, *C. imicola*, *C. punctatus*, *C. newsteadi*, *C. lupicaris* and *C. pulicaris*) did not feed on other hosts but on cattle by multiplex-PCR assays. Although there were other animals on the farms, the fact that they prefer only cattle can be attributed to the low number of other hosts on the farms. It may also be due to cattle being more attractive hosts for *Culicoides* species found in the farms in this study.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (A. Deniz) on reasonable request.

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Ethical Approval

Not necessary

Competing Interests

The authors declared that there is no conflict of interest

Author Contributions

The study was designed by AD and CK. Fly samples were collected by AD, CK, HB, AİE and OS. Culicoides species identification was made by CK, UE and ST. Multiplex-PCR diagnosis was made by AD, HB and ZV. The original draft was prepared by AD. All authors contributed to the review and proofreading of the article.

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