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Molecular Characterization of *Culicoides* Species and Their Vector Potentials for Haemosporidia Infections in the İzmir Region of Türkiye

Türkiye'nin İzmir Yöresinde Yayılış Gösteren Culicoides Türlerinin Moleküler Karakterizasyonu ve Haemosporidia Enfeksiyonları Yönünden Vektörlük Potansiyelleri

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ABSTRACT

Objective: This study aimed to determine the *Culicoides* species distributed across different districts of İzmir province, reveal their molecular characterization, and assess their vector potential for the transmission of avian haemosporidian parasites.
Methods: The study material comprised 800 female *Culicoides* specimens collected from Bergama, Ödemiş, Kemalpaşa, and Foça districts between May and August 2016. Following morphological identification, specimens from each identified species underwent molecular analyses. The *mt-COI* gene region of genomic DNA isolates from the specimens was amplified by polymerase chain reaction (PCR) and subjected to sequence analyses to reveal their molecular characterization and phylogenetic relationships. Haemosporidian DNA was investigated by nested PCR in the gDNA isolates of head/thorax (HTP) and abdomen pools, constituted from specimens separated by species and location. Molecular characterization of identified parasites was performed using sequence analyses.
Results: Morphological identification revealed that *C. circumscriptus* (39.4%) and *C. imicola* (33.8%) were the most common species in the research areas, followed by *Culicoides* sp. (ERU-Izm-Culi1) (9.1%), *C. nubeculosus* complex (7.6%), *C. obsoletus* (4.3%), *C. gejgelensis* (2.3%), *C. punctatus* (1.9%), and *C. newsteadi* (1.8%). A total of 175 polymorphic sites were distributed among the COI sequences of the obtained isolates, leading to the detection of 18 different haplotypes. The highest haplotype diversity was observed in *C. circumscriptus*, *C. punctatus*, and *C. newsteadi*. Phylogenetic analyses clustered the characterized haplotypes of *Culicoides* species into three major groups. *Haemoproteus* sp. GAGLA05 and *H. minutus* TURDUS2 lineages were detected in *C. circumscriptus* HTP genomic DNA isolates, providing evidence of this species' vector potential for *Haemoproteus* lineages in the research area.
Conclusion: This study determined the *Culicoides* species distributed in the İzmir Region using an integrated morphological and molecular diagnostic approach, providing original data for the molecular epidemiology of these important flies. Furthermore, the results suggest the potential importance of *C. circumscriptus* in the transmission dynamics of *Haemoproteus* lineages.
Keywords: *Culicoides*, molecular characterization, avian Haemosporidians, vector potential, İzmir

ÖZ

Amaç: Bu çalışmada İzmir'in farklı ilçelerinde yayılış gösteren *Culicoides* türlerinin belirlenmesi, moleküler karakterizasyonlarının yapılması ve kanatlı haemosporidian parazitleri yönünden vektörlük potansiyellerinin araştırılması amaçlanmıştır.
Yöntemler: Çalışma materyalini, 2016 yılının Mayıs-Ağustos ayları arasında Bergama, Ödemiş, Kemalpaşa ve Foça ilçelerinden toplanan toplam 800 adet dişi *Culicoides* örneği oluşturmuştur. *Culicoides* örneklerinin morfolojik teşhislerini takiben belirlenen her türe ait örnekler moleküler analizlere dahil edilmiştir. Örneklere ait genomik DNA izolatlarının *mt-COI* gen bölgesi polimeraz zincir reaksiyonunda (PZR) amplifiye edilmiş ve sonraki basamakta amplifikasyon ürünlerinin sekans analizleri gerçekleştirilerek moleküler karakterizasyonları ve filogenetik analizleri yapılmıştır. Tür ve lokasyonuna göre ayrılan örneklerin baş/toraks (BTH) ve abdomenlerinden ayrı havuzlar oluşturularak elde edilen gDNA izolatlarında haemosporidian DNA'sının varlığı nested PZR ile araştırılmış ve tespit edilen parazit nesillerinin sekans analizleri ile moleküler karakterizasyonları yapılmıştır.

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Türkiye PARAZİTOLOJİ Dergisi

Bulgular: Morfolojik teşhis sonuçlarına göre araştırma bölgelerinde *C. circumscriptus* (%39,4) ve *C. imicola*'nın (%33,8) en yaygın türler olduğu bunu sırasıyla *Culicoides* sp. (ERU-Izm-Culi1) (%9,1), *C. nubeculosus* kompleks (%7,6), *C. obsoletus* (%4,3), *C. gejgelensis* (%2,3), *C. punctatus* (%1,9) ve *C. newsteadi*'nin (%1,8) izlediği saptanmıştır. Belirlenen türlere ait mt-COI sekansları arasında 18 farklı haplotipi ortaya koyan 175 polimorfik bölge saptanmış olup haplotip çeşitliliği en yüksek *C. circumscriptus, C. punctatus* ve *C. newsteadi*'de belirlenmiştir. Filogenetik analizlerde belirlenen *Culicoides* türlerine ait haplotipler üç major küme içerisinde monofiletik olarak gruplanmıştır. *Culicoides circumscriptus* BTH'ye ait genomik DNA izolatlarında *Haemoproteus* sp. GAGLA05 ve *H. minutus* TURDUS2 nesillerinin varlığı saptanmış ve bu türün araştırma yöresinde ilgili *Haemoproteus* nesillerinin muhtemel potansiyel vektörü olabileceği ortaya çıkarılmıştır.
Sonuç: Bu çalışma ile İzmir yöresinde yaygınlık gösteren *Culicoides* türleri entegre morfolojik ve moleküler teşhis yaklaşımlarıyla belirlenerek bu önemli sineklerin moleküler epidemiyolojisi açısından özgün veriler sağlanmıştır. Ayrıca çalışma sonuçları İzmir yöresinde *C. circumscriptus*'un *Haemoproteus* nesillerinin nakli açısından potansiyel bir öneme sahip olduğuna dair kanıtlar sağlamıştır.
Anahtar Kelimeler: *Culicoides*, moleküler karakterizasyon, kanatlı Haemosporidian parazitler, vektör potansiyeli, İzmir

INTRODUCTION

Culicoides Latreille, 1809 (Diptera: Ceratopogonidae), also known as biting midges, are the smallest blood-feeding insects with a body length of 1 to 3 mm among other biting insects (1). The genus *Culicoides* is important due to their ability to act as biological vectors for pathogens of medical and veterinary significance (2). Several nematode and protozoan species, as well as more than 50 arboviruses, have been isolated from various *Culicoides* species, and their roles in the transmission of veterinary (1,3,4) and human pathogens (5) have been reviewed. Currently, the most important economic impact of *Culicoides* flies is their association with the transmission of diseases such as Blue Tongue virus (BTV), Epizootic Hemorrhagic Disease virus, Schmallenberg virus, and African Horse Sickness virus. These arboviruses are highly significant for ruminants, deer, and ungulates, and have been reported by the World Organization for Animal Health to cause outbreaks (6,7).

Culicoides flies are the main vectors of *Haemoproteus* (Haemosporida) species, which are important parasites of birds and can cause lethal pathology in non-adapted birds (8-11). Despite more than 1360 *Culicoides* species have been known (12), completeness of sporogonic phase of avian *Haemoproteus* parasites have been shown only in 13 species which indicates those serve as active vectors (13,14). In addition to about 150 *Haemoproteus* species identified from birds (15). In a study conducted in Türkiye to investigate haemosporidian parasites in *Culicoides* species (16), various *Haemoproteus* lineages were molecularly characterized in specimens of *C. nubeculosus* comp., *C. riethi*, *C. circumscriptus*, *C. submaritimus*, *C. gejgelensis*, *C. longipennis*, *C. festivipennis*, and *C. newsteadi* collected from the Sultan Marshes Region. The data obtained from this study (16) provided information on the vectorial potentials of the relevant *Culicoides* species.

The first records on *Culicoides* species in Türkiye were provided by Kieffer (17), and data on species diversity have mainly increased after the 1970s. In a review by Dik (18), the number of *Culicoides* species in Türkiye was reported as 61. Later, Korkmaz et al. (19) identified new species through a comprehensive four-year research conducted in 51 provinces at 104 sampling stations, increasing the total number of species to 72 (20). Limited research has been conducted on the genetic diversity of *Culicoides* species in Türkiye. Dik et al. (21) characterized the *Culicoides* species collected from the Southern and Southeastern Anatolian Regions based on the ribosomal *ITS-1* gene region. The characterization of *Culicoides* species collected from Sultan Marshes (16), the Western Black Sea Region, and Konya (22,23) was conducted based on the *mitochondrial cytochrome oxidase subunit I* (*mt-COI*) gene region, and their phylogenetic relationships were determined. Korkmaz et al. (19) characterized the *mt-COI* gene region of the *C. grisescens* and *C. chiopterus* species, that were reported for the first time in Türkiye.

In the current study, we aimed to determine the *Culicoides* species prevalent in different districts of İzmir province using conventional morphological and molecular methods, to perform molecular characterization of the isolates, and to reveal their vector potentials for the transmission of avian haemosporidian parasites.

METHODS

Study Area and Collection of *Culicoides* Specimens

The *Culicoides* specimens comprising the study material were collected from areas in the districts of Bergama, Ödemiş, Kemalpaşa, and Foça, located within the boundaries of İzmir province, between May and August 2016. "EVS Black CO2 Light Trap, 4W Black Light Tube (Bioquip Inc., 2801BL)" and Onderstepoort light traps were set up approximately 1.5-2 meters high, one hour before sunset, and operated until one hour after sunrise in livestock facilities in the corresponding districts. After sampling, the nets of the light traps were stored in a deep freezer to immobilize the caught flies. Non-target flies were separated macroscopically, and the remaining flies were placed in 70% alcohol and transported to the laboratory on ice packs. From each region, 200 samples were randomly selected and included in the study.

Morphological Identification

Identification of the *Culicoides* specimens was made based on wing morphology using a combination of established and more recent diagnostic keys (24-26). Examinations were conducted under a digital camera attachment stereomicroscope (Olympus SZX-16). The imaging of the wing morphologies of *Culicoides* species was performed using CellSens Standard 1.13 software (Olympus) under the camera attachment stereomicroscope.

Genomic DNA Extraction

Morphologically identified *Culicoides* specimens were separated individually from the collection material preserved in 70% ethyl alcohol for genotyping and phylogenetic analyses. The remaining specimens were pooled in groups of ten (10 samples/pool) to investigate the presence of Haemosporidian parasites, including females, according to species and collection area. To demonstrate the vector potential of *Culicoides* specimens, the heads/thoraxes (HTP) (sporozoite development) and abdomens (AP) (haemosporidian generations acquired through blood feeding) of each specimen were dissected separately and placed in separate sterile microcentrifuge tubes and pooled accordingly,

resulting in 88 pools each for HTP and AP. Genomic DNA isolation was performed using the GeneJET Genomic DNA Purification Kit (Thermo Scientific) following the manufacturer's instructions.

Amplification of *Culicoides* Mt-COI and Avian Haemosporidian *Mt-CYTB* Gene Regions

Genomic DNA extracts isolated from individual female *Culicoides* specimens were subjected to polymerase chain reaction (PCR) analysis using primers C1-J-1718M (F) (5'-GGAGGATTTGGAAATTGATTAGT-3') and C1-N-2191M (R) (5'-CAGGTAAAATTAAAATATAAACTTCDGG-3'), which amplify a 523 bp partial fragment of the *mt-COI* gene of the *Culicoides* genus (27). The reaction mixture was prepared to a final volume of 25 µL. It contained 10X PCR buffer, 2.5 mM $MgCl_2$, 100 nM of each primer, 200 µM of each dNTP, 2.5 U Taq DNA polymerase, and 10-20 ng template DNA. The thermal cycling protocol was as follows: Initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s, with a final extension step at 72 °C for 7 min.

The genomic DNA isolates obtained from Culicoides HTP and AP were analyzed by Nested PCR targeting a 524 bp portion of the *mt-CYTB* gene region of haemosporidian parasites. In the first step of the Nested PCR analysis, universal primers HaemNFI (5'-CATATATTAAGAGAATATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') were used for haemosporidians. In the second step, *Leucocytozoon*-specific primers HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATGGIGC-3'), and *Plasmodium/Haemoproteus*-specific primers HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') were used (28,29). The reaction mixture for both primer sets was prepared to a final volume of 20 µL, containing 10X PCR buffer, 2.5 mM $MgCl_2$, 100 nM of each primer, 200 µM of each dNTP, and 2.5U Taq DNA polymerase. The PCR products were subjected to electrophoresis on a 1.5% agarose gel (10 µL), visualized and analyzed using the CLP Gel Documentation System and Gene Snap from Syngene analysis program (UVP INC Uplant, CA).

Sequence and Phylogenetic Analyses

The amplicons of mt-COI from individual *Culicoides* specimens and haemosporidian mt-cytb from pools were subjected to gel purification (using the High Pure PCR Product Purification Kit, Roche) for sequence analyses. Sequencing was utilized (Macrogen Europe) bi-directionally with the primers used in the PCR (the second primer pair for haemosporidian mt-CYTB). Chromatograms for forward and reverse reads were processed with DeNovo Assembly in Geneious R10 (30) software to obtain consensus sequences. The molecular characterizations of the identified isolates were achieved by aligning the sequences with the published sequences of the isolates using BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the MalAvi Haemosporidian lineage sequence database (http://mbio-serv2.mbioekol.lu.se/Malavi/), and data sets were created for phylogenetic analyses. The *mt-COI* gene sequences of the identified *Culicoides* isolates in the study were recorded in the GenBank database with accession numbers MF594384-MF594401; and the mt-CYTB sequences of haemosporidian isolates were recorded with accession numbers MF594402-MF594403. DnaSP 5.10.01 (31) software was used to determine DNA polymorphism and haplotype diversity in the characterized *Culicoides* isolates. Intra- and inter-specific genetic differences were performed using the Kimura (32) two-parameter (K2P) distance model in MEGA 7 software (33). Bayesian inference (BI) analyses were applied to determine the phylogenetic relationships of *Culicoides* species and haemosporidian lineages. jModelTest v.0.1.1 (34) was used to determine the best-fit substitution model for sequence evolution, and the models with the lowest Akaike Information Criterion values were used to construct the phylogenetic trees. BI analyses were performed using the MrBayes (35) plugin in Geneious R10 (30) software. Markov Chain Monte Carlo scans were run for 1,100,000 generations with four chains, and tree sampling was performed every 200 generations with the first 100,000 trees discarded as "burn-in".

Statistical Analysis

Statistical analysis is not required for the data available in this study.

RESULTS

Culicoides Species and Distribution

The distribution of the morphologically identified *Culicoides* specimens by species, collection area, and month are shown in Table 1. A total of eight *Culicoides* species were identified in the research areas, and their distribution rates are shown in Figure 1. Generally, the most common species were *C. circumscriptus* (39.4%) and *C. imicola* (33.8%), while the species with the lowest distribution rates were *C. punctatus* (1.9%) and *C. newsteadi* (1.8%).

A unique *Culicoides* lineage was identified that exhibited a similar wing morphology to the species in the Silvaticulicoides subgenus (36,37), and was genetically closer to the species in this group. This lineage was characterized as *Culicoides* sp. ERU-Izm-Culi1. The specimens of *Culicoides* sp. ERU-Izm-Culi1 were detected in Bergama and Ödemiş districts in July and August samples (Table 1), and the overall distribution rate was determined to be 9.1% (Figure 2).

Sequence and Phylogenetic Analyses of *Culicoides* Specimens

Genomic DNA isolates of the specimens identified by morphological identification were amplified with the relevant primers for the *mt-COI* gene region, and target amplicons of 553 bp were obtained. The morphological diagnosis results were confirmed by aligning the sequences of the relevant isolates with reference isolates in GenBank using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the molecular characterization of the isolates was provided. The isolates belonging to the species whose *mt-COI* gene region sequences and haplotype characterization were determined were given in Table 2 with GenBank accession numbers. One hundred seventy five polymorphic regions were found among the mt-COI sequences of the identified species, revealing 18 different haplotypes, and the average genetic diversity among haplotypes was found to be 25.3%±2.4. The haplotype diversity was determined to be highest in *C. circumscriptus, C. punctatus,* and *C. newsteadi* (Table 2). ERU-Izm-Culi1 was found to be genetically closer to species under the

Table 1. Distribution of *Culicoides* species collected from various districts of İzmir province by collection sites and months

***Culicoides* species**	**Collection area**				**2016**				**Total**
	Bergama	**Ödemiş**	**Kemalpaşa**	**Foça**	**May**	**June**	**July**	**August**	
C. circumscriptus	46	82	112	75	68	128	38	81	315
C. nubeculosus komp.	40	18	0	3	22	6	9	24	61
C. newsteadi	14	0	0	0	14	0	0	0	14
C. imicola	27	53	70	120	80	56	65	69	270
Culicoides sp. (*ERU-Izm-Culi1*)	58	15	0	0	0	0	65	8	73
C. gejgelensis	8	0	8	2	0	0	4	14	18
C. obsoletus	7	17	10	0	16	0	16	2	34
C. punctatus	0	15	0	0	0	10	3	2	15
Total	200	200	200	200	200	200	200	200	800

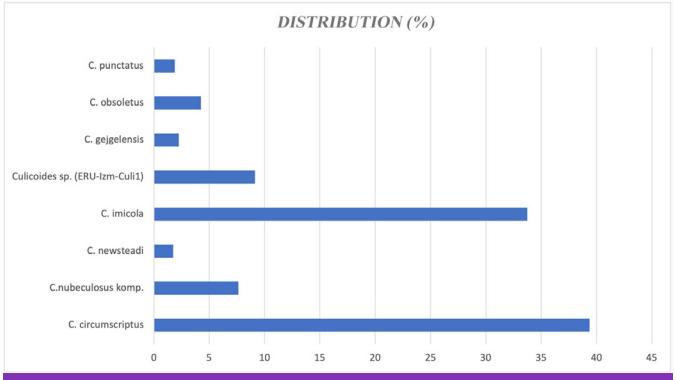

Figure 1. Distribution of *Culicoides* species identified in the study area

Figure 2. *Culicoides* sp. ERU-Izm-Culi1 wing morphology. There are no white spots in the middle of cells m1 (1) and m2 (2), the m cell is combined with the r-m cross-vein white spots (3), and the white spots on the distal parts of cells r3 and m1 are not clear (4). Scale bar: 200 µm

Silvaticulicoides subgenus, showing the highest genetic similarity of 92.9% with *C. subfasciipennis*.

The phylogenetic relationships of the identified isolates in this study along with reported isolates from various regions of Türkiye and the world, were shown in Figure 3. The mt-COI sequences formed three major clusters on the phylogenetic tree, containing monophyletic species, and this resolution was highly supported by posterior probabilities (0.9-1.0). The first major cluster was divided into two subclusters. The first subcluster included *C. nubeculosus, C. gejgelensis, C. riethi, Culicoides* sp. ERU-Izm-Culi1, and *C. subfasciipennis* species, and the phylogenetic relationship between the species was moderately supported by posterior probability (0.58). The second subcluster was formed by *C. circumscriptus*, and the phylogenetic relationship of the isolates belonging to this species was highly supported by posterior probability (1.0). The second major cluster consisted of haplotypes of *C. punctatus, C. newsteadi-1*, and *C. newsteadi-2* species, which were highly supported by posterior probability (0.92). The third major cluster included *C. obsoletus* and *C. imicola* species, and the phylogenetic relationship of the isolates belonging to these species was highly supported by posterior probability (1.0).

Identification, Distribution, and Molecular Characterization of Haemosporidian Parasites in *Culicoides* Specimens

The molecular analysis results of haemosporidian parasites in *Culicoides* specimens, which were collected during the study and diagnosed by morphological and molecular identifications, are provided in Table 3 for HTP and AP samples. An agarose gel image including the amplicons from some positive samples is given in Figure 4. Of the 88 AP samples examined, 9 (10.22%) were found to be infected with *Plasmodium/Haemoproteus* species or lineages, and 7 (7.95%) of the 88 HTP samples were also found to be infected with these parasites. No positivity for *Leucocytozoon* sp. was found in any of the samples.

The haemosporidian parasite species or lineages identified by sequence analysis along with their GenBank accession numbers, are given in Table 4. The GenBank accessions were provided only for isolates identified in the head/thorax pools as an indicator of their vector potential. Of the seven positive isolates from *C. circumscriptus* HTP, four belonged to the *Haemoproteus* sp. GAGLA05 lineage, and three belonged to the *H. minutus* TURDUS2 lineage. All of the positive AP isolates were characterized by *Plasmodium* lineages, and this result could be related to infected avian blood in their abdomens. The latest finding provided evidence of the widespread occurrence of various *Plasmodium* lineages, especially the *P. relictum* SGS1, in avian populations in the study area.

Table 2. Haplotypes identified in *Culicoides* species molecularly characterized by *Mt-COI* gene region sequence analyses

***Culicoides* species**	**No. of sequenced isolates**	**Determined haplotype**		
		Name	**Number**	**GenBank accession**
C. circumscriptus	6	ERU-Izm-C.circ1	3	MF594384
		ERU-Izm-C.circ2	2	MF594385
		ERU-Izm-C.circ3	1	MF594386
C. gejgelensis	7	ERU-Izm-C.gejg1	7	MF594387
C. imicola	6	ERU-Izm-C.imic1	4	MF594388
		ERU-Izm-C.imic2	2	MF594389
C. newsteadi	7	ERU-Izm-C.news1	4	MF594390
		ERU-Izm-C.news2	2	MF594391
		ERU-Izm-C.news3	1	MF594392
C. nubeculosus	6	ERU-Izm-C.nube1	5	MF594393
		ERU-Izm-C.nube2	1	MF594394
C. riethi	1	ERU-Izm-C.rie1	1	MF594400
C. obsoletus	13	ERU-Izm-C.obs1	6	MF594395
		ERU-Izm-C.obs2	7	MF594396
C. punctatus	6	ERU-Izm-C.punc1	4	MF594397
		ERU-Izm-C.punc2	1	MF594398
		ERU-Izm-C.punc3	1	MF594399
Culicoides sp.	7	ERU-Izm-Culi1	7	MF594401

Table 3. Molecular analysis results of Haemosporidian parasites in examined *Culicoides* specimens

Insect species	**No. of examined pools**										***Plasmodium/Haemoproteus* positivity**									
	Bergama		**Ödemiş**		**Kemalpaşa**		**Foça**		**Total**		**Bergama**		**Ödemiş**		**Kemalpaşa**		**Foça**		**Total**	
	AP	**HTP**	**AP**	**HTP**	**AP**	**HTP**	**AP**	**HTP**	**AP**	**HTPT**	**AP**	**HTP**	**AP**	**HTP**	**AP**	**HTP**	**AP**	**HTP**	**AP**	**HTP**
C. circumscriptus	5	5	7	7	10	10	7	7	29	29	1	1	0	1	0	3	0	2	1	7
C. imicola	3	3	6	6	7	7	11	11	27	27	0	0	0	0	1	0	2	0	3	0
Culicoides sp. (close to *C. truncorum*)	6	6	2	2	0	0	0	0	8	8	0	0	0	0	0	0	0	0	0	0
C. nubeculosus comp.	4	4	3	3	0	0	1	1	8	8	0	0	0	0	0	0	0	0	0	0
C. obsoletus	1	1	3	3	3	3	0	0	7	7	1	0	2	0	0	0	0	0	3	0
C. gejgelensis	1	1	0	0	2	2	1	1	4	4	0	0	0	0	2	0	0	0	2	0
C. punctatus	0	0	3	3	0	0	0	0	3	3	0	0	0	0	0	0	0	0	0	0
C. newsteadi	2	2	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0
Total	22	22	24	24	22	22	20	20	88	88	2	1	2	1	3	3	2	2	9	7

DISCUSSION

The molecular genotyping and DNA barcoding studies of blood-sucking insects with medical and veterinary importance such as mosquitoes, biting midges, and black flies continue to gain importance in many regions of the world (38-41), while genetic studies in Türkiye have been limited (21,22,42,43). The *Culicoides* genus is important because it contains many species that are biological vectors for numerous pathogens of medical and veterinary importance (1,4,5,36). In the province of İzmir, due to its climatic characteristics (high temperature and humidity), the circulation of BTV is continuous in the field because *Culicoides* activity continues for a long period of the year. Therefore, it has been reported that BTV disease causes significant losses in the livestock sector in the province of İzmir every year (44). In this study, field surveys conducted in the regions of Bergama, Ödemiş, Kemalpaşa, and Foça districts of İzmir between May and August revealed that those regions were suitable ecosystems for the development of *Culicoides* species. As a result of the morphological and molecular diagnoses of *Culicoides* generations collected from the sampling areas, it was determined that *C. circumscriptus* and *C. imicola* were the most frequently encountered species during the

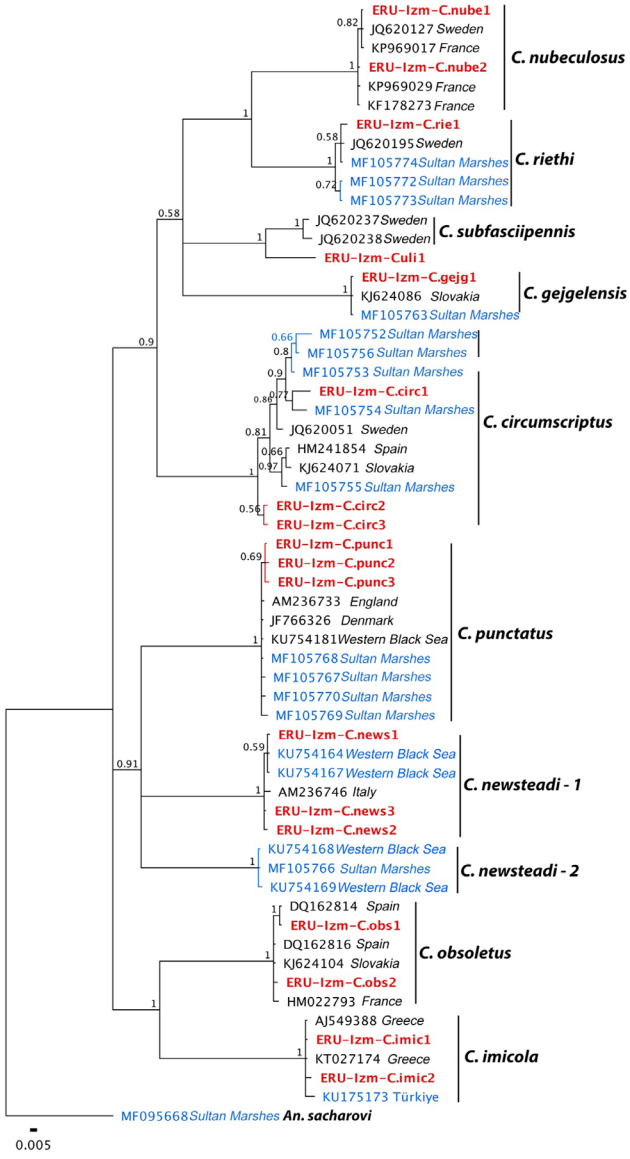

Figure 3. Phylogenetic relationships of *Culicoides* isolates based on *mt-COI* gene region Bayesian inference (BI) analysis. The isolates identified in the study are shown in bold red characters, while those reported from Türkiye are shown in blue. The numbers in front of the nodes represent BI posterior probabilities. *A. sacharovi* was used as an outgroup. The scale bar indicates the number of nucleotide substitutions per site

study period, representing 39.4% and 33.8% of the total collected specimens, respectively. In particular, it is known that *C. imicola* is one of the most effective vectors of BTV in many geographies, especially in Southern Europe (45,46), and it may play a role in the transmission dynamics of BTV observed in the research areas in the current study. Studies on populations sampled from Türkiye (47) and Italy (48) have suggested that *C. circumscriptus*, which was identified as the most frequently encountered species in our study, could also potentially contribute to the transmission dynamics of BTV. In research conducted in the last 10 years (21,22,47,49-54), both species have been reported as widespread

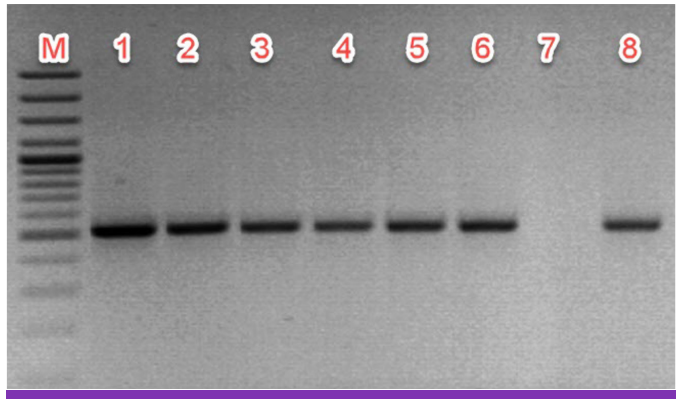

Figure 4. Agarose gel electrophoresis of *Haemoproteus/Plasmodium* amplicons obtained from Nested polymerase chain reaction analysis of gDNA isolates from selected *Culicoides* pools. M: 100 bp marker; 1-6: positive samples; 7: Negative control; 8: Positive control (Plasmodium DNA)

in Southern and Southeastern Anatolia, Central and Western Black Sea, Central Anatolia, Western Inner Anatolia, and the Marmara Region in Türkiye. The other species *C. nubeculosus* complex (7.6%), *C. obsoletus* (4.3%), *C. gejgelensis* (2.3%), *C. punctatus* (1.9%), and *C. newsteadi* (1.8%), which have a more limited distribution in our study, were found in various regions of Türkiye such as Southern and Southeastern Anatolia, Central and Western Black Sea, Central Anatolia, Western Inner Anatolia, and the Marmara Region (21,22,47,49-54). A unique *Culicoides* lineage was identified in Bergama and Ödemiş districts. This lineage showed similarities to members of the Silvaticulicoides subgenus based on wing morphology and was genetically closer to the species in this group based on mt-COI sequence characterization. The wing morphologies of these specimens were consistent and symmetrical across all samples. Furthermore, sequence analysis of a representative number of specimens confirmed the genetic similarity within this group. There was no genetic heterogeneity among the mt-COI sequences of samples belonging to this Culicoides lineage, and this group was designated as Culicoides sp. ERU-Izm-Culi1.

Based on the phylogenetic analysis of the mt-COI gene sequences of the isolates identified in our study, the characterized haplotypes were monophyletically placed in three major clusters. Low genetic diversity (0.2±0.2%) was identified between *C. nubeculosus* ERU-Izm-C.nube1-2 haplotypes within the first sub-cluster of the first major cluster, with ERU-Izm-C.nube1 haplotype being 100% identical to isolates from Sweden (JQ620127) and France (KP969017, 55), and ERU-Izm-C.nube2 haplotype being 100% identical to isolates from France (KP969029, KF178273, 55, 56). The ERU-Izm-C.rie1 haplotype, identified within the *C. nubeculosus* complex and molecularly characterized as belonging to *C. riethi*, formed a cluster showing high identity (99.6% and 99.4% respectively) with isolates reported from Sultan Marshes, Türkiye (MF105774, 16) and Sweden (JQ620237). This isolate also showed 99.1% identity with other *C. riethi* isolates reported from Sultan Marshes, Türkiye (MF105772, MF105773, 16). The *C. gejgelensis* ERU-Izm-C.gejg1 haplotype showed 100% identity with isolates reported from Sultan Marshes, Türkiye (MF105763, 16) and Slovakia (KJ624086, 37), and formed a cluster together. The mt-COI sequences of the specimens characterized as *Culicoides* sp. ERU-Izm-C.culi1 showed 100% identity to each

Table 4. Molecular characterization of Haemosporidian parasites according to the sequence analysis results

Species	No. of sequenced isolates		Sequence characterization of Haemosporidia			
	AP	HTP	*Plasmodium* species/ lineage	Number (AP)	*Haemoproteus* species/ lineage	Number (HTP) GenBank accession
Culicoides						
C. circumscriptus	1	7	*Plasmodium* sp./CXPIP10	1	*H. minutus*/TURDUS2	3 (MF594402)
					Haemoproteus sp./GAGLA05	4 (MF594403)
C.imicola	3	0	*P. relictum*/SGS1	2	-	-
			Plasmodium sp./YWT4	1		
C. obsoletus	3	0	*P. relictum*/SGS1	1	-	-
			Plasmodium sp./CXPIP23	1		
			Plasmodium sp./SYCON02	1		
C. gejgelensis	2	0	*P. relictum*/SGS1	1	-	-
			Plasmodium sp./YWT4	1	-	-

*: Sequence data obtained only from the BT pools were deposited in GenBank and accession numbers were provided for Haemosporidian species and lineages based on their vector potential, AP: Abdomen, HTP: Head/thorax

other, thus representing the same haplotype. The ERU-Izm-C. culi1 haplotype showed the highest genetic similarity (92.9%) with *C. subfasciipennis* isolates reported from Sweden (JQ620237, JQ620238), and formed a cluster together. This *Culicoides* lineage was closer to the species in the Silvaticulicoides subgenus with more than 7% genetic differences than to other *Culicoides* species. Therefore, the related *Culicoides* lineage may be an unidentified new species, and further studies are needed for species identification. The isolates of *C. circumscriptus* in the second sub-cluster of the first major cluster were found to form three separate groups. The ERU-Izm-C.circ1 haplotype showed high identity (average 97.3%) and formed a cluster with isolates from Sweden and Sultan Marshes, Türkiye (JQ620051, MF105754, 16). One isolate each from Spain (HM241854), Slovakia (KJ624071, 37), and Sultan Marshes, Türkiye (MF105755, 16) were included in the second group. A genetic difference of 0.4±0.3% was identified between the ERU-Izm-C.circ2 and ERU-Izm-C.circ3 haplotypes, and these two haplotypes formed the third cluster within the relevant group.

The second major group was formed by haplotypes belonging to *C. punctatus, C. newsteadi-1*, and *C. newsteadi-2* species. The clustering of the *C. newsteadi-1* and *2* species were made according to the classification of Pages et al. (25). A genetic difference of 0.3±0.2 was detected between the ERU-Izm-C.punc1-3 haplotypes *of C. punctatus*, and these haplotypes exhibited mean similarities of 99.5%, 99.7%, 99.4% and 99.6% to *C. punctatus* isolates from Sultan Marshes, Türkiye (MF105768-70, 16), Western Black Sea (KU754181, 22), England (AM236733, 24), and Denmark (JF766326, 57), respectively. Phylogenetic analysis of *C. newsteadi* revealed two separate groups, one of which was close to *C. punctatus*. ERU-Izm-C.news1-3 haplotypes were found in the first phylogenetic group, with the ERU-Izm-C. news1 haplotype grouping with the Western Black Sea isolates (KU754164, KU754167, 22) with 100% identity. The ERU-Izm-C. news2,3 haplotypes showed the highest identity with ERU-Izm-C. news1 and again with the Western Black Sea isolates (KU754164, KU754167, 22) (avg. 99.7%). The characterized isolates were also found to be on average 99.5% identical to the *C. newsteadi* isolate reported from Italy (AM236746, 24) in the same phylogenetic group. Isolates from the Western Black Sea (KU754168, KU754169, 22) and Sultan Marshes, Türkiye (MF105766, 16) comprised the second phylogenetic group of *C. newsteadi*, and this group showed an average genetic difference of 19.5±2.7% with the first phylogenetic group of *C. newsteadi*.

In the third major cluster, *C. obsoletus* and *C. imicola* are present. There is a genetic difference of 0.6±0.4% between the ERU-Izm-C. obs1 and ERU-Izm-C.obs2 haplotypes of *C. obsoletus*. The ERU-Izm-C.obs1 haplotype is identical to the isolate reported from Spain (DQ162814, 57) and is grouped accordingly. The ERU-Izm-C.obs2 haplotype has the highest similarity of 99.8% with the isolates from Spain (DQ162814, 57) and Slovakia (KJ624104, 36). *C. imicola* has a genetic difference of 0.4±0.3% between the haplotypes ERU-Izm-C.imic1 and ERU-Izm-C.imic2. The ERU-Izm-C.imic1 haplotype is 100% identical to the isolates from Greece (KT027174, 58), while the ERU-Izm-C.imic2 haplotype has a 99.6% similarity with the same isolates. Furthermore, the characterized haplotypes have shown similarities of 99.4% and 98.9% with the isolate reported from the Konya Region of Türkiye (KU175173).

In this study, as various researchers have indicated (59,60), to determine the infective and infected *Culicoides* specimens with haemosporidian parasites in the research area, pools were formed by dissecting the head-thorax (potentially infective) and abdomen (potentially infected) of each fly diagnosed at the species level. As is known, infective stage sporozoites are transmitted through the salivary glands of the female fly during feeding. The salivary glands extend towards the esophagus on the lateral sides of the thorax (61). Fertilization and oocyst development (non-infective

stage) take place in the mid-gut part (abdomen) of the fly. It has been expressed in various studies (59,60,62) that head-thorax pool (HTP) positivity can be considered an indicator of potential vector competence. Indeed, in our study, sequence analyses of the positive isolates obtained from HTP confirmed the presence of *Haemoproteus* parasites that *Culicoides* species are known to vector. In contrast, the sequences obtained from abdomen pools (AP) showed sequences of *Plasmodium* parasites, which are likely not vectored by *Culicoides*, possibly due to infected blood intake.

The *Haemoproteus* sp. GAGLA05 lineage identified in *C. circumscriptus* has been recorded in the blood of the Eurasian jay (*Garrulus glandarius*) belonging to the Passeriformes order (63), and parallel to our study, it was also isolated from *C. circumscriptus* in the Sultan Marshes Region of Türkiye (16). The *H. minutus* TURDUS2 lineage has been found to be common in avian communities, reported from 17 bird species belonging to eight families in the Passeriformes order and one bird species in the Psittaciformes order (MalAvi: http://mbio-serv2.mbioekol.lu.se/Malavi/). Additionally, the TURDUS2 lineage has been reported in mosquito species such as *Cx. pipiens* (64) and in *Culicoides* species including *C. festivipennis*, *C. kibunensis* (65), and *C. pictipennis* (66). Our study has revealed the first record of the potential vector status of *C. circumscriptus* for the possible transmission of the *H. minutus* TURDUS2 lineage. The *Plasmodium* sp. CXPIP10 lineage isolated and characterized from the abdominal pools of *C. circumscriptus* in our study has been reported in MalAvi records as a potential vector by various *Culex* species (67,68). The *Plasmodium* sp. CXPIP23 lineage isolated from *C. obsoletus* has been reported in the Little Bittern (*Ixobrychus minutus*) belonging to the Ciconiiformes order in the Sultan Marshes Region, Türkiye (16) and potential mosquito vectors *Cx. pipiens* (16) and *O. caspius* (62). The *P. relictum* SGS1 lineage identified in AP from *C. gejgelensis, C. imicola*, and *C. obsoletus* is a common lineage in birds and has been isolated from numerous bird species in 11 orders. *Plasmodium* sp. YWT4 isolated from *C. imicola* and *C. gejgelensis* AP and *Plasmodium* sp. SYCON02 from *C. obsoletus* AP have been reported in a limited number of bird species in the Passeriformes order up to the present day (MalAvi: http://mbio-serv2.mbioekol.lu.se/Malavi/). The results obtained in this study have revealed the prevalence of the relevant *Plasmodium* lineages in avian communities in the İzmir Region and that *Culicoides* species feed on infected hosts.

CONCLUSION

Culicoides species that are prevalent in the İzmir Region have been morphologically and molecularly characterized, and unique epidemiological data have been provided based on molecular ecology regarding the vector potential of these species for avian haemosporidian parasites. The identification of species, such as *C. imicola*, known as the active vector for the BTV, highlights the need for control and management strategies for this disease in the context of host-pathogen-vector. Furthermore, the results of this study have shown that molecular-based techniques, in conjunction with morphometric analyses, can reliably determine species diversity of *Culicoides* in different geographies in Türkiye, and can also reveal cryptic or sibling species in epidemiological context. Considering the different biogeographical characteristics in Türkiye, there is a need for detailed studies on *Culicoides* species and the diseases they transmit based on molecular epidemiology.

*Ethics

Ethics Committee Approval: No need to get ethics approval due to the material used in the study were insect specimens.

Informed Consent: Not required.

Peer-review:

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Footnotes

*Authorship Contributions

Concept: H.Y., Z.Ö., Design: H.Y., Z.Ö., Data Collection or Processing: H.Y., Z.Ö., A.Y., Analysis or Interpretation: H.Y., Z.Ö., Literature Search: H.Y., Z.Ö., A.Y., Writing: H.Y., Z.Ö., A.Y.

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REFERENCES

1. Mellor PS, Boorman J, Baylis M. *Culicoides* biting midges: their role as arbovirus vectors. Annu Rev Entomol. 2000; 45: 307-40.
2. Harrup LE, Bellis GA, Balenghien T, Garros C. *Culicoides* Latreille (Diptera: Ceratopogonidae) taxonomy: current challenges and future directions. Infect Genet Evol. 2015; 30: 249-66.
3. Borkent A. The biting midges, the Ceratopogonidae (Diptera), biology of disease vectors, 2nd ed. Elsevier, Burlington, MA: USA; 2004: 113-126.
4. Meiswinkel R, Venter GJ, Nevill EM. Vectors: *Culicoides* spp. In: Coetzer JAW, Tustin RC, editors. Infectious Diseases of Livestock. Cape Town: Oxford University Press; 2004: 93-136.
5. Carpenter S, Groschup MH, Garros C, Felippe-Bauer MF, Purse B. *Culicoides* biting midges, arboviruses and public health in Europe. Antiviral Res. 2013; 13: 102-13.
6. OIE. OIE-Listed diseases, infections and infestations in force in 2014, updated: 1st January 2014, accessed: 17th January 2014. Available at: http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2014/.
7. Elbers AR, Meiswinkel R, van Weezep E, van Oldruitenborgh-Oosterbaan MM, Kooi EA. Schmallenberg virus in *Culicoides* spp. biting midges, the Netherlands, 2011. Emerg Infect Dis. 2013; 19: 106-9.
8. Valkiūnas G. Avian malaria parasites and other haemosporidia. Boca Raton: CRC Press; 2005.
9. Atkinson CT. *Haemoproteus*. In: Atkinson CT, Thomas NJ, Hunter BC, editors. Parasitic diseases of wild birds. Ames: Wiley-Blackwell; 2008. p.13-35.
10. Donovan TA, Schrenzel M, Tucker TA, Pessier AP, Stalis IH. Hepatic hemorrhage, hemocoelom, and sudden death due to *Haemoproteus* infection in passerine birds: eleven cases. J Vet Diagn Invest. 2008; 20: 304-13.
11. Ortiz-Catedral L, Brunton D, Stidworth MF, Elsheikha HM, Pennycott T, Schulze C, et al. *Haemoproteus minutus* is highly virulent for Australasian and South American parrots. Parasit Vector. 2019; 12: 40.
12. Borkent A. World species of biting midges (Diptera: Ceratopogonidae). New York: American Museum of Natural History; 2019.
13. Bukauskaitė D, Iezhova TA, Ilgūnas M, Valkiūnas G. High susceptibility of the laboratory-reared biting midges *Culicoides nubeculosus* to *Haemoproteus* infections, with review on *Culicoides* species that transmit avian haemoproteids. Parasitology. 2019; 146: 1-9.

14. Bernotienė R, Žiegytė R, Vaitkutė G, Valkiūnas G. Identification of a new vector species of avian haemoproteids, with a description of methodology for the determination of natural vectors of haemosporidian parasites. Parasites & Vectors. 2019; 12: 307.
15. Iezhova TA, Dodge M, Sehgal RN, Smith TB, Valkiunas G. New avian *Haemoproteus* species (Haemosporida: Haemoproteidae) from African birds, with a critique of the use of host taxonomic information in hemoproteid classification. J Parasites. 2011; 97: 682-94.
16. Yıldırım A, Dik B, Düzlü Ö, Gürsoy Ergen A. Sultan sazlığı ekosisteminde kanatlı Haemosporidialarının moleküler ekolojisi. TÜBİTAK 1001 Projesi, Proje no: 114 O 646, 2017.
17. Kieffer JJ. Chironomides d'Afrique et d'Asie conservés au Museum National Hongrois de Budapest. Ann Hist Nat Mus Nat Hung. 1918; 16: 31-136.
18. Dik B. *Culicoides* (Diptera: Ceratopogonidae) vektörlükleri ve mücadelesi. İçinde: Özbel Y (editör), Vektör artropodlar ve mücadelesi. Türkiye Parazitoloji Derneği: İzmir; 2017. s.46-122.
19. Korkmaz C, Alten B, Erol U, Deniz A. Updated checklist of *Culicoides* Latreille (Diptera: Ceratopogonidae) of Türkiye with ten new records. J Vector Ecol. 2021; 46: 124-36.
20. Turgut F. Morphological characteristics of two *Culicoides* Latreille biting midges (Diptera, Ceratopogonidae) with a new record for Turkish fauna. Trakya Univ J Nat Sci. 2023; 23: 3-8.
21. Dik B, Muz D, Muz MN, Uslu U. The geographical distribution and first molecular analysis of *Culicoides* Latreille (Diptera: Ceratopogonidae) species in the Southern and Southeastern Türkiye during the 2012 outbreak of bovine ephemeral fever. Parasitol Res. 2014; 113: 4225-32.
22. Yildirim A, Dik B, Duzlu O, Onder Z, Ciloglu A, Yetismis G, et al. Genetic diversity of *Culicoides* species within the Pulicaris complex (Diptera: Ceratopogonidae) in Türkiye inferred from mitochondrial COI gene sequences. Acta Trop. 2019; 190: 380-8.
23. Şevik M, Oz ME. Detection of Peste des petits ruminants virus RNA in *Culicoides imicola* (Diptera: Ceratopogonidae) in Türkiye. Vet Ital. 2019; 55: 173-9.
24. Nolan DV, Carpenter S, Barber J, Mellor PS, Dallas JF, Mordue Luntz AJ, et al. Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. Vet Microbiol. 2007; 124: 82-94.
25. Pages N, Munoz-Munoz F, Talavera S, Sarto V, Lorca C, Núñez JI. Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides* and development of species-specific PCR assays based on barcode regions. Vet Parasitol. 2009; 165: 298-310.
26. Mathieu B, Cêtre-Sossah C, Garros C, Chavernac D, Balenghien T, Carpenter S, et al. Development and validation of IIKC: an interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the Western Palaearctic region. Parasites & Vectors. 2012; 5: 137.
27. Dallas JF, Cruickshank RH, Linton YM, Nolan DV, Patakakis M, Braverman Y, et al. Phylogenetic status and matrilineal structure of the biting midge, *Culicoides imicola*, in Portugal, Rhodes and Israel. Med Vet Entomol. 2003; 17: 379-87.
28. Hellgren O, Waldenström J, Bensch S. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol. 2004; 90: 797-802.
29. Bensch S, Hellgren O, Pérez-Tris J. MalAvi: A public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol Ecol Resour. 2009; 9: 1353.
30. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012; 28: 1647-9.
31. Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009; 25: 1451-2.
32. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980; 16: 111-20.
33. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013; 30: 2725-9.
34. Posada D. jModelTest: phylogenetic model averaging. Mol Biol Evol. 2008; 25: 1253-6.
35. Huelsenbeck JP, Ronquist F. MRBAYES: bayesian inference of phylogenetic trees. Bioinformatics. 2001; 17: 754-5.
36. Ramilo DW, Nunes T, Madeira S, Boinas F, da Fonseca IP. Geographical distribution of *Culicoides* (Diptera: Ceratopogonidae) in mainland Portugal: presence/absence modelling of vector and potential vector species. PLoS One. 2017; 12: e0180606.
37. Sarvašová A, Kočišová A, Halán M, Delécolle JC, Mathieu B. Morphological and molecular analysis of the genus *Culicoides* (Diptera: Ceratopogonidae) in Slovakia with five new records. Zootaxa. 2014; 3872: 541-60.
38. Carvalho LPC, Costa GDS, Pereira Júnior AM, de Paulo PFM, Silva GS, Carioca ALPM, et al. DNA Barcoding of genus *Culicoides* biting midges (Diptera: Ceratopogonidae) in the Brazilian Amazon. Acta Trop. 2022; 235: 106619.
39. Gopurenko D, Bellis G, Pengsakul T, Siriyasatien P, Thepparat A. DNA barcoding of *Culicoides* Latreille (Diptera: Ceratopogonidae) from Thailand reveals taxonomic inconsistencies and novel diversity among reported sequences. J Med Entomol. 2022; 59: 1960-70.
40. Ortega-Morales AI, Hernández-Triana LM, Chan-Chable RJ, Garza-Hernández JA, González-Álvarez VH, Ruiz-Arrondo I, et al. DNA barcoding of mosquitoes from the Pantanos de Centla biosphere reserve, Southeastern Mexico. J Am Mosq Control Assoc. 2021; 37: 198-207.
41. Dähn O, Werner D, Mathieu B, Kampen H. Large-scale cytochrome C Oxidase Subunit I gene data analysis for the development of a multiplex polymerase chain reaction test capable of identifying biting midge vector species and haplotypes (Diptera: ceratopogonidae) of the *Culicoides* Subgenus *Avaritia* Fox, 1955. Genes (Basel). 2024; 15: 323.
42. Onder Z, Yildirim A, Duzlu O, Arslan MO, Sari B, Tasci GT, et al. Molecular characterization of black flies (Diptera: Simuliidae) in areas with pest outbreaks and simuliotoxicosis in Northeast Anatolia Region, Türkiye. Acta Trop. 2019; 199: 105149.
43. Inci A, Yildirim A, Duzlu O, Onder Z, Ciloglu A, Seitz G, et al. Genetic diversity and identification of Palearctic black flies in the subgenus Wilhelmia (Diptera: Simuliidae). J Med Entomol. 2017; 54: 888-94.
44. Anon. İzmir Bornova Veteriner Kontrol Enstitüsü Kayıtları; 2016.
45. Goredo M, Catalani M, Federici V, Portanti O, Marini V, Mancini G, et al. Vector species of *Culicoides* midges implicated in the 2012-2014 Bluetongue epidemics in Italy. Vet Ital. 2015; 51: 131-8.
46. Goredo M, Meiswinkel R, Federici V, Di Nicola F, Mancini G, Ippoliti C, et al. The "*Culicoides obsoletus* group" in Italy: Relative abundance, geographic range, and role as vector for Bluetongue virus. Vet Ital. 2016; 52: 235-24.
47. Yavru S, Dik B, Bulut O, Yavru N, Kale M, Bulut A. New *Culicoides* vector species for BTV transmission in Central and Central west of Anatolia. Annu Res Rev Biol. 2018; 27: 1-9.
48. Foxi C, Meloni G, Puggioni G, Manunta D, Rocchigiani A, Vento L, et al. Bluetongue virus detection in new *Culicoides* species in Sardinia, Italy. Vet Re. 2019; 184: 621.
49. Dik B, Kuçlu Ö, Öztürk R. *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) species in the Western Black Sea region of Türkiye, new records for the Turkish fauna. Turk J Vet Anim Sci. 2017; 41: 228-37.
50. Reyhan E. Güney Doğu Anadolu Bölgesinin Gaziantep, Kahramanmaraş ve Kilis illerinde *Culicoides* (Diptera: Ceratopogonidae) türlerinin yayılışı, Yüksek Lisans Tezi, Gaziantep Üniversitesi Fen Bilimleri Enstitüsü: Gaziantep; 2018.
51. Turgut F, Kilic AY. The Ceratopogonidae (Insecta: Diptera) fauna of the Central Black Sea region in Türkiye. Turk Zool Derg. 2015; 39: 1071-89.
52. Muz D, Dik B, Muz MN, Öztürk R. Tekirdağ ili *Culicoides* (Diptera: Ceratopogonidae) faunasının mavi dil virus ve Schmallenberg virus varlığı yönünden araştırılması. Namık Kemal Üniversitesi Araştırma Projesi: 2016, Proje no: NKUBAP.00.23.AR.14.08.

53. Turgut F. Samsun ili *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) faunasına katkılar. GBAD. 2018; 7: 14-20.

54. Turgut F. Contributions to *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) fauna of Sinop province. SAUJS. 2018; 22: 1683-93.

55. Hadj-Henni L, De Meulemeester T, Mathieu B, Depaquit J, Augot D. Taxonomic assessment of *Culicoides brunnicans*, *C. santonicus* and *C. vexans* (Diptera: Ceratopogonidae) in France: Implications in systematics. Infect Genet Evol. 2015; 33: 324-31.

56. Augot D, Ninio C, Akhoundi M, Lehrter V, Couloux A, Jouet D, et al. Characterization of two cryptic species, *Culicoides stigma* and *C. parroti* (Diptera: Ceratopogonidae), based on barcode regions and morphology. J Vector Ecol. 2013; 38: 260-5.

57. Lassen SB, Nielsen SA, Skovgård H, Kristensen M. Molecular differentiation of *Culicoides* biting midges (Diptera: Ceratopogonidae) from the subgenus *Culicoides* Latreille in Denmark. Parasitol Res. 2012; 110: 1765-71.

58. Pagès N, Sarto I Monteys V. Differentiation of *Culicoides obsoletus* and *Culicoides scoticus* (Diptera: Ceratopogonidae) based on mitochondrial cytochrome oxidase subunit I. J Med Entomol. 2005; 42: 1026-34.

59. Ishtiaq F, Guillaumot L, Clegg SM, Phillimore AB, Black RA, Owens IP, et al. Avian haematozoan parasites and their associations with mosquitoes across Southwest Pacific Islands. Mol Ecol. 2008; 17: 4545-55.

60. Njabo KY, Cornel AJ, Bonneaud C, Toffelmier E, Sehgal RN, Valkiūnas G, et al. Nonspecific patterns of vector, host and avian malaria parasite associations in a central African rainforest. Mol Ecol. 2011; 20: 1049-61.

61. James AA, Rossignol PA. Mosquito salivary glands: parasitological and molecular aspects. Parasitol Today. 1991; 7: 267-71.

62. Inci A, Yıldırım A, Njabo KY, Duzlu O, Biskin Z, Ciloglu A. Detection and molecular characterization of avian *Plasmodium* from mosquitoes in central Türkiye. Vet Parasitol. 2012; 188: 179-84.

63. Mata VA, da Silva LP, Lopes RJ, Drovetski SV. The strait of gibraltar poses an effective barrier to host-specialised but not to host-generalised lineages of avian Haemosporidia. Int J Parasitol. 2015; 45: 711-9.

64. Martinez-de la Puente J, Munoz J, Capelli G, Montarsi F, Soriguer R, Arnoldi D, et al. Avian malaria parasites in the last supper: identifying encounters between parasites and the invasive Asian mosquito tiger and native mosquito species in Italy. Malaria J. 2015; 14: 32.

65. Synek P, Munclinger P, Albrecht T, Votypka J. Avian haemosporidians in haematophagous insects in the Czech Republic. Parasitol Res. 2013; 112: 839-45.

66. Bobeva A, Zehtindjiev P, Bensch S, Radrova J. A survey of biting midges of the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) in NE Bulgaria, with respect to transmission of avian haemosporidians. Acta Parasitol. 2013; 58: 585-91.

67. Ferraguti M, Martinez-de la Puente J, Munoz J, Roiz D, Ruiz S, Soriguer R, et al. Avian *Plasmodium* in *Culex* and *Ochlerotatus* mosquitoes from Southern Spain: effects of season and host-feeding source on parasite dynamics. PLoS One. 2013; 8: e66237.

68. Kim KS, Tsuda Y. Avian *Plasmodium* lineages found in spot surveys of mosquitoes from 2007 to 2010 at Sakata wetland, Japan: do dominant lineages persist for multiple years? Mol Ecol. 2012; 21: 5374-85.