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Development of Conventional Multiplex PCR Assays for the Identification of 21 West Palaearctic Biting Midge Taxa (Diptera: Ceratopogonidae) Belonging to the *Culicoides* Subgenus *Culicoides*, including Recently Discovered Species and Genetic Variants

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Abstract: In 2006 and 2011, two biting-midge-borne arboviruses of high veterinary importance emerged for the first time in Central Europe: bluetongue virus (BTV) and Schmallenberg virus (SBV). Members of the native Obsoletus and Pulicaris Groups were soon identified as the potential vectors. However, despite several years of extensive taxonomic research on these groups, correct species identification and differentiation from closely related species are still challenging due to isomorphic features, the existence of cryptic species and obsolete PCR identification assays. At present, 17 valid West Palaearctic biting midge species of the *Culicoides* subgenus *Culicoides*, including the Pulicaris Group, are known, and additional genetic variants have been described. For many of them, no identification tests are available, and their roles in disease transmission have remained unknown. In this study, 465 GenBank DNA sequence entries of the mitochondrial cytochrome c oxidase subunit I (COI) gene were used to design PCR primers as specific genetic markers for 21 West Palaearctic biting midge taxa of the *Culicoides* subgenus *Culicoides*. During their validation with DNA from field-collected biting midges and synthetic DNA from biting midge genotypes not available from the field, all primers detected their target taxa, while few showed cross-reactions. Our results indicate the great potential of the new primers in PCR assays and clearly demonstrate the suitability of the COI gene as an excellent marker for the identification of different biting midge species and genetic variants of the *Culicoides* subgenus *Culicoides*.

Keywords: *Culicoides*; Pulicaris Group; West Palaearctic; vectors; polymerase chain reaction (PCR); mitochondrial cytochrome c oxidase subunit I (COI)

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1. Introduction

Biting midges of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) are considered the smallest hematophagous dipterans on our planet [1], capable of transmitting a variety of nematodes, protozoan parasites and viruses to domestic animals, an unknown number of wild animal species and—in rare cases—humans [2,3]. Despite their high abundance in Europe, the early isolation of African horse sickness virus (AHSV) from mixed pools of the widely distributed Obsoletus and Pulicaris Groups [4,5] and the detection of bluetongue virus (BTV) in field-collected *C. obsoletus* specimens [4], little attention had been given to indigenous biting midge species for many years. In 2006, the unprecedented outbreak of bluetongue disease (BT) in European areas where the Afro-Asian vector species

C. imicola was absent changed this attitude and highlighted the importance of Palaearctic biting midges in pathogen transmission. Subsequent entomological surveillance confirmed the long-suspected involvement of species of both the Obsoletus Group (genus *Culicoides*, subgenus *Avaritia* Fox, 1955) and the Pulicaris Group (genus *Culicoides*, subgenus *Culicoides* Latreille, 1809) in the transmission of BTV [6–14] and, a few years later, also associated them with the newly emerged Schmallenberg virus (SBV) [15–26]. However, for most species, vector competence has so far been suggested rather than experimentally proven, and correct species identification is challenging, especially in the heterogeneous subgenus *Culicoides*, to which the Pulicaris Group belongs.

To date, 17 valid species of the subgenus *Culicoides* have been described for the western Palaearctic: *C. almeidae* Cambournac, 1970; *C. boyi* Nielsen, Kristensen and Pape, 2015; *C. bysta* Sarvašová and Mathieu, 2017; *C. cryptipulicaris* Talavera, Muñoz-Muñoz, Verdún and Pagès, 2017; *C. delta* Edwards, 1939/*C. lupicaris* Downes and Kettle, 1952 (considered synonymous); *C. fagineus* Edwards, 1939; *C. flavipulicaris* Dzhafarov, 1964; *C. grisescens* Edwards, 1939/*C. remmi* Damien-Georgescu, 1972 (considered synonymous); *C. impunctatus* Goetghebuer, 1920; *C. kalix* Nielsen, Kristensen and Pape, 2015; *C. newsteadi* Austen, 1921; *C. paradoxalis* Ramilo and Delécolle, 2013; *C. pulicaris* (Linnaeus, 1758); *C. punctatus* (Meigen, 1804); *C. quasipulicaris* Talavera, Muñoz-Muñoz, Verdún and Pagès, 2017; *C. selandicus* Nielsen, Kristensen and Pape, 2015; and *C. subfagineus* Delécolle and Ortega, 1998 [27]. However, the taxonomy of the subgenus *Culicoides* is apparently far more complex, and the elucidation of biting midge phylogeny must be seen as an ongoing process [2].

Confusingly, a variety of synonyms are commonly used for one and the same species in the subgenus *Culicoides*: e.g., *C. pulicaris* is also known as *C. setosinervis* Kieffer, 1913; *C. pullatus* Kieffer, 1915; *C. stephensi* Carter, 1916; *C. cinerellus* Kieffer, 1919; *C. quinquepunctatus* Goetghebuer, 1921; *C. flaviplumus* Kieffer, 1924; and *C. sawamotoi* Kono and Takahasi, 1940 [27]. In some cases, it has been further discussed whether synonyms, for example, *C. delta*/*C. lupicaris* and *C. grisescens*/*C. remmi*, should be considered separate species [2,28–31].

The identification of biting midges is classically based on morphological features, particularly the wing pigmentation of adult insects, which allows a quick separation into the Obsoletus Group, Pulicaris Group and other *Culicoides* spp. [32,33]. This method proves to be difficult for the identification to the species level of females of closely related species that have very similar or identical (isomorphic) features and is even more challenging for juvenile specimens (larvae, pupae) [34–36], for which either identification keys have not yet been developed or distinguishing features are not yet even established for corresponding adults. Furthermore, morphological species identification cannot be used for the determination of phylogenetic distances [37] and may require time-consuming analyses of slide-mounted microscopical insect preparations to visualize fine structures [38], a lot of practical experience and fresh material with distinct coloration.

The development and implementation of molecular tools such as species-specific PCR tests and DNA barcoding have improved the knowledge of phylogenetic relationships and revolutionized the species identification of biting midges. For these genetic techniques, various molecular markers have been used, such as mitochondrial and nuclear genes, including ribosomal markers [39]. The mitochondrial cytochrome oxidase c subunit I (COI or COX1) gene has been by far the most widely utilized marker for phylogenetic studies and identification purposes in culicoid biting midges, as it is a sufficiently long high-copy gene that is composed of both conserved and variable regions [40–42].

In the past two decades, comprehensive studies of the COI region have revealed a considerable number of genetic variants in the subgenus *Culicoides*: Pagès et al. [43] described a new haplotype of *C. pulicaris* (*C. pulicaris* haplotype P3) from Spain, which was described later as *C. cryptipulicaris* [31], a previously unknown haplotype of *C. fagineus* (referred to as *C. fagineus* haplotype F1), and three new genetic variants of *C. newsteadi* (haplotypes N1, N2 and N3). These findings were supported by COI analyses of biting midges collected in Denmark and Sweden [44]. Similar heterogeneity in the mitochondrial

gene sequence was found in *C. grisescens* specimens from Switzerland (haplotypes G1 and G2) [45] and in *C. lupicaris* biting midges from various European countries, including Spain, Denmark, Czech Republic, France, Slovakia, Sweden, Turkey and Austria (*C. lupicaris* sensu stricto (s.s.), *C. lupicaris* haplotypes L2 and L3) [30,43,44,46–49]. Additionally, another genotype that is morphologically similar to *C. fagineus* haplotype F1—*Culicoides* WBS—was recently reported from the Black Sea region of Turkey [48], and it is expected that further genetic variants will be discovered in the future [43,46,47].

Several PCR tests have been designed to distinguish between common West Palaearctic species of the subgenus *Culicoides* and some of their genetic variants known at that time [34,43,45,50]. However, these PCR tests are incapable of differentiating newly discovered haplotypes and were developed more than ten years ago using a small and spatially restricted gene pool, which limits their applicability. Commonly used COI barcoding is not an appropriate alternative for species identification, as it cannot be applied to pooled samples due to the risk of mixed taxa/sequences and the consequent detection of the more abundant species. Moreover, previous analyses of engorged females led to the unintended identification of the blood-donor species and failed to characterize individuals stored in ethanol for extended time periods. Thus, a revision of published PCR tests and the development of new diagnostic assays are urgently necessary.

In the present study, a huge dataset of West Palaearctic subgenus *Culicoides* COI gene sequences from GenBank was analyzed with the aim to develop easy-to-use multiplex PCR assays for the differentiation of their species and genetic variants. The COI gene features both variable and conserved regions and is represented in GenBank by a number of entries sufficient to provide comprehensive and reliable information on DNA variations and homologies between species and haplotypes. Some authors, however, have already addressed the issue of wrong entries in such data repositories [46,51,52], which are mainly regarded as attributable to the preceding incorrect morphological identification of specimens. Since classical taxonomists are becoming progressively scarce all the while cryptic taxa are being detected, reliable alternative techniques have to be developed, thus providing the basis for the improved identification of potential vector species and a better understanding of the *Culicoides* biting midge distribution and ecology.

2. Materials and Methods

2.1. Biting Midge Collection

Culicoid biting midges were collected with BG-Sentinel UV-light suction traps (Biogents, Regensburg, Germany) operated once a week for 24 h during various German monitoring activities. The individual specimens analyzed originated from samplings in other European countries. Biting midges were morphologically pre-identified under a stereomicroscope to the group or species level using commonly used identification keys [38,53–55]. Pre-sorted biting midges were kept in 75% EtOH for subsequent molecular analysis.

2.2. Genetic Identification of Field-Collected Biting Midges

After discarding the ethanol and evaporating the remaining fixative for 1 min at room temperature, three steel beads with a diameter of 3 mm (TIS GmbH, Gauting, Germany) were added to morphologically pre-identified, single specimens of the subgenus *Culicoides*. The samples were supplemented with either 180 µL of buffer ATL and 20 µL of Proteinase K (Qiagen, Hilden, Germany) or 350 µL of in-house ZB5d medium (Eagle's minimal essential medium with Earle's and Hank's salts plus non-essential amino acids) containing 3.5 µL of penicillin–streptomycin (100 U/mL) and 0.7 µL of gentamycin–amphotericin (0.01 mg/mL, 0.25 µg/mL) (Thermo Fisher Scientific, Dreieich, Germany). Samples were homogenized for 3 min at 30 Hz with a TissueLyser II (Qiagen), and total DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) or the NucleoMag VET Kit (Macherey-Nagel, Düren, Germany) according to the manufacturers' instructions, with a final elution volume of 50 µL of AE-buffer (QIAamp DNA Mini Kit) or 100 µL of VEL-buffer (NucleoMag VET Kit), respectively.

DNA extracts were used to generate amplicons of the COI gene with the species-specific PCR described in Nolan et al. [50], the universal primers PanCuli-COX1-211F and PanCuli-COX1-727R according to the authors' protocol [56] or the self-designed generic primer PanCuli-COX1-025F (5′-ACTTTATATTTTATTTTTGGAGYWTGRGC-3′) in combination with PanCuli-COX1-727R using an adapted protocol (54 °C annealing temperature) from Lehmann et al. [56]. PCR products with expected lengths were excised and extracted with the QIAquick Gel Extraction Kit (Qiagen). For sequencing, DNA fragments were cycled with the PCR primers using the BigDye Termintor v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The resulting PCR products were purified with the Bioanalysis NucleoSEQ Kit (Macherey-Nagel), and 15 µL of the eluates were mixed with the same volume of Hi-Di formamide (Thermo Fisher Scientific). Each sample was sequenced on a 3500 Genetic Analyzer (Applied Biosystems/Hitachi, Darmstadt, Germany), followed by sequence editing with Geneious Prime software version 2021.0.1 (Biomatters, Auckland, New Zealand). Edited sequences were deposited in GenBank.

2.3. COI Data Analysis and Primer Design

All available GenBank entries of West Palaearctic taxa of the subgenus *Culicoides* were collected and checked for plausibility: COI sequences (Table S1) were compared with sequences of the first description to find incorrect entries. Dubious sequences were re-analyzed with the NCBI nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 28 October 2022)), assigned to fitting species or—if no sequence match could be found—excluded from further analysis. Remaining sequences were used for the generation of consensus sequences with Geneious Prime software (Biomatters), which were finally compared in a Geneious multiple alignment using initial settings. In this context, it is to be noted that no sequences were found in GenBank under the species name '*C. delta*'. Instead, sequences of that taxon had been deposited using the name '*C. deltus*', which had been used for '*C. delta*' until renaming in 2015.

Inter- and intraspecific variances in the DNA sequence were used to design specific forward primers, including wobble sites, according to common guidelines for primer design [57–59]. Promising primer candidates were checked regarding melting temperature, GC content, self-dimerization and primer-dimer formation with the Oligo Analysis Tool (https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/ (accessed on 14 December 2021)) and analyzed with the NCBI BLAST tool for repetitive sequences before ordering. Primers were checked for functionality, specificity and the capability of multiplexing and finally validated with genetically identified biting midge material from the field or—in case no field-collected material of the respective taxon was available—synthetic COI gene DNA (Table S2), produced by GenExpress (Berlin, Germany).

2.4. Multiplex PCRs

The newly designed specific forward primers were applied in combination with the published universal reverse primer PanCuli-COX1-727R [56] in several multiplex PCRs (mPCRs), which can be carried out in parallel or successively. For easier handling, the approach was based on a universal annealing temperature instead of primer-specific annealing temperatures, although this increased the risk of reduced primer sensitivity and specificity. As far as possible, the primers were combined in the various mPCRs according to morphological similarities of the species or haplotypes they were meant to detect, but this was not possible in all cases.

The master mixes were composed of 10 µL of 2× QuantiTect Multiplex PCR NoROX reagent (Qiagen), 0.5 µM of each primer and 2 µL of DNA template and replenished with water to give a total volume of 20 µL. DNA amplification was performed using the following thermoprofile: 15 min at 95 °C (activation of Taq polymerase), followed by 42 cycles of 30 s at 95 °C (denaturation), 45 s at 63 °C (primer annealing) and 45 s at 72 °C (primer elongation), and a final elongation step for 5 min at 72 °C. The complete PCR reaction mixture was supplemented with 2.5 µL of 6× DNA Loading Dye (Thermo Fisher

Scientific) and applied to 1.5% agarose gels, pre-mixed with 5 mg/mL ethidium bromide solution. After running for 50 min at 100 V, gels were visualized with a ChemiDoc MP Imaging System (Bio-Rad, Feldkirchen, Germany).

3. Results

During the analysis of the comprehensive GenBank dataset of COI sequences from 21 different countries, a significant number of subgenus *Culicoides* sequences was found to be incorrectly deposited, especially those of specimens with similar morphology (e.g., *C. newsteadi* group). However, implausible entries could be identified, and a total number of 465 COI sequences could accurately be assigned to the respective species and haplotypes (Table S1).

For each species and haplotype, specific consensus sequences with lengths between 412 and 1535 base pairs were generated, which revealed high intraspecific pairwise identity between 98.3% and 99.8% (average: 99.4%, Table S1). Multiple alignment comparison of the consensus sequences displayed interspecific differences between 6.0% and 19.7% (Figure 1). Interestingly, genetic distances between synonymous *C. delta*/*C. lupicaris* taxa and recently described haplotypes of the same species, especially *C. newsteadi*, were comparatively high (10.0% to 18.0%), questioning their taxonomic status. Thus, these taxa were considered separate taxonomic entities during PCR development.

Inter	pul	cry	del	lup L1	pun	imp	kal	boy	bys	new N3	lup L2	fla	new N1	gri G1	sel	fag F1	fag F2	sub	new N2	gri G2	new
pul		89.1	86.1	85.9	85.5	85.0	84.7	84.5	84.4	84.2	84.2	84.0	83.8	83.1	82.9	82.8	82.7	82.7	82.6	82.1	81.8
cry	10.9		84.7	85.0	85.6	83.4	83.1	80.3	80.8	82.5	85.4	82.2	83.8	81.2	82.8	82.8	83.2	81.5	83.0	80.3	81.4
del	13.9	15.3		86.7	85.1	84.9	87.0	85.3	84.1	85.3	84.9	83.5	83.3	84.2	85.6	82.5	84.1	82.8	83.9	82.9	84.6
lup L1	14.1	15.0	13.3		84.2	85.7	84.9	85.8	85.1	83.6	85.1	83.6	84.3	82.1	85.0	81.8	83.6	84.0	84.3	83.0	84.8
pun	14.5	14.4	14.9	15.8		84.8	89.0	84.0	83.8	82.3	86.7	85.3	85.5	85.3	85.9	80.8	82.6	83.7	84.3	83.8	84.6
imp	15.0	16.6	15.1	14.3	15.2		86.2	85.9	84.8	86.7	87.1	83.4	83.4	83.8	83.5	81.3	83.6	82.5	84.1	81.7	84.7
kal	15.3	16.9	13.0	15.1	11.0	13.8		85.1	84.2	84.6	85.7	84.1	84.5	85.4	93.9	83.0	84.3	85.1	84.1	81.8	85.5
boy	15.5	19.7	14.7	14.2	16.0	14.1	14.9		94.0	85.3	84.5	83.8	84.4	82.8	84.3	84.7	82.6	83.3	82.4	84.6	86.2
bys	15.6	19.2	15.9	14.9	16.2	15.2	15.8	6.0		83.8	85.1	83.4	84.7	82.6	83.4	82.8	83.4	81.3	81.7	83.2	85.4
new N3	15.8	17.5	14.7	16.4	17.7	13.3	15.4	14.7	16.2		86.9	83.8	82.3	82.5	83.8	83.3	84.6	84.1	84.5	82.2	85.7
lup L2	15.8	14.6	15.1	14.9	13.3	12.9	14.3	15.5	14.9	13.1		83.7	83.7	84.1	84.5	81.8	84.1	82.8	84.3	82.0	84.9
fla	16.0	17.8	16.5	16.4	14.7	16.6	15.9	16.2	16.6	16.2	16.3		85.7	85.9	85.0	88.3	89.1	87.0	81.7	86.1	85.4
new N1	16.2	16.2	16.7	15.7	14.5	16.6	15.5	15.6	15.3	17.7	16.3	14.3		84.3	84.8	81.1	83.7	82.7	82.0	83.4	85.6
gri G1	16.9	18.8	15.8	17.9	14.7	16.2	14.6	17.2	17.4	17.5	15.9	14.1	15.7		84.1	83.7	85.3	85.2	81.1	90.0	82.1
sel	17.1	17.2	14.4	15.0	14.1	16.5	6.1	15.7	16.6	16.2	15.5	15.0	15.6	15.9		82.5	83.8	83.5	82.8	82.5	85.4
fag F1	17.2	17.2	17.5	18.2	19.2	18.7	17.0	15.3	17.2	16.7	18.2	11.7	18.9	16.3	17.5		89.8	89.9	80.8	85.0	82.3
fag F2	17.3	16.8	15.9	16.4	17.4	16.4	15.7	17.4	16.6	15.4	15.9	10.9	16.3	14.7	16.2	10.2		89.1	84.1	85.4	84.8
sub	17.3	18.5	17.2	16.0	16.3	17.5	14.9	16.7	18.7	15.9	17.2	13.0	17.3	14.8	16.5	10.1	10.9		81.8	84.8	83.3
new N2	17.4	17.0	16.1	15.7	15.7	15.9	15.9	17.6	18.3	15.5	15.7	18.3	18.0	18.9	17.2	19.2	15.9	18.2		81.5	83.2
gri G2	17.9	19.7	17.1	17.0	16.2	18.3	18.2	15.4	16.8	17.8	18.0	13.9	16.6	10.0	17.5	15.0	14.6	15.2	18.5		83.5
new	18.2	18.6	15.4	15.2	15.4	15.3	14.5	13.8	14.6	14.3	15.1	14.6	14.4	17.9	14.6	17.7	15.2	16.7	16.8	16.5	
Intra	0.3	0.2	0.4	0.5	0.7	0.3	0.6	0.2	1.7	0.3	0.2	0.2	0.9	0.2	0.7	0.3	0.9	0.7	1.0	1.1	0.3
n	82	4	14	31	105	30	14	13	21	19	9	7	16	42	7	3	14	6	7	6	15

Figure 1. Inter- and intraspecific pairwise genetic comparison of COI gene DNA sequences between tested West Palaearctic taxa of the subgenus *Culicoides*: genetic distances are displayed in the left-bottom half of the matrix and highlighted with graded colors from red (low distance) through yellow (medium distance) to green (high distance). Genetic similarities are presented in the right-upper half of the matrix. Values (in %) were calculated through the comparison of species- and haplotype-specific consensus sequences of respective GenBank entries (n). *C. pulicaris* (pul), *C. cryptipulicaris* (cry), *C. delta* (del), *C. lupicaris* haplotype L1 (lup L1), *C. punctatus* (pun), *C. impunctatus* (imp), *C. kalix* (kal), *C. boyi* (boy), *C. bysta* (bys), *C. newsteadi* haplotype N3 (new N3), *C. lupicaris* haplotype L2 (lup L2), *C. flavipulicaris* (fla), *C. newsteadi* haplotype N1 (new N1), *C. grisescens* haplotype G1 (gri G1), *C. selandicus* (sel), *C. fagineus* haplotype F1 (fag F1), *C. fagineus* haplotype F2 (fag F2), *C. subfagineus* (sub), *C. newsteadi* haplotype N2 (new N2), *C. grisescens* haplotype G2 (gri G2) and *C. newsteadi* s.s. (new).

Genetic differences were subsequently used to develop specific forward primers for 21 West Palaearctic biting midge taxa of the *Culicoides* subgenus *Culicoides* according to the PCR concept published in Lehmann et al. [56] (Table 1). On average, 14 primers per species (290 in total, Table S3) were tested, and in many cases, pre-testing revealed cross-reactivity

with other subgenus *Culicoides* taxa. However, the targeted insertion of wobbles and mismatch bases significantly reduced unspecific detection. The best-performing forward primers (Table 1), in combination with PanCuli-COX1-727R as a reverse primer [56], were put together in various single-tube mPCRs. Merely the number of species and haplotypes to be considered and the limited length of generated consensus sequences made it necessary to subdivide the PCR approach into four reactions (mPCRs A–D). The mPCRs were pre-tested with DNA extracts of single specimens or with an equivalent of 10^6 copies of synthetic COI gene DNA (calculated based on serial dilution quantitative real-time PCR) of subgenus *Culicoides* taxa to verify multiplexability (Figure 2A–D). Each multiplex PCR showed the expected amplicons of the target species between 139 bp and 491 bp, whereas no amplification was observed for no-template negative controls (Figure 2, lanes 2, 9, 15 and 21).

Table 1. Newly designed forward primers specific for 21 taxa of the *Culicoides* subgenus *Culicoides* to be used in combination with the universal reverse primer PanCuli-COX1-727R.

mPCR	Species/Haplotype	Primer Code	Primer Sequence (5′-3′)	Modification (Position)	Amplicon (bp)
A	*C. bysta*	bys-COI-158F	AATCTTACTTCTCTTATCTCTRC	R-wobble (2)	158
	C. punctatus	pun-COI-227F	TCATATGCGATCAAACGGG	A > C (18)	227
	C. boyi	boy-COI-275F	AGCTATTTCATCAATTCTTGGA	G > C (20)	275
	C. grisescens G2	gri2-COI-346F	CCACACCTTTCTGCAAACA	C > A (15)	346
	C. kalix	kal-COI-419F	CCACCCTTCTCTAACATTGC	C > A (18)	419
	C. grisescens G1	gri1-COI-463F	GATATAGCTTTCACACGAATG	C > A (9)	463
B	*C. fagineus* F2	fag2-COI-151F [3]	TTGCATCTTTCCCTCCCTGTA	T > A (17)	151
	C. flavipulicaris	fla-COI-215F [3]	CAATCGTATTACTTTTGATCGT	G > C (18)	215
	C. subfagineus	sub-COI-318F [3]	CTGTRGCTTCTGTAGATC	R-wobble (14), G > T (15)	318
	C. fagineus F1	fag1-COI-420F	TTCCTCCATCTCTTTCCCTAT	C > T (17)	420
	C. impunctatus	imp-COI-491F	ATTGGTTCCATTAATACTCGGA	none	491
C	*C. delta*	del-COI-161F	TGCTATATTACTTCTTTTGTCAC	T > A (17)	161
	C. lupicaris L1 [1]	lup1-COI-214F	AATGGAATGTCATTCGACCGT	T > G (13)	214
	C. pulicaris s.s.	pul-COI-313F [2,3]	GCATCCGTAGACTTGGCC	none	313
	C. cryptipulicaris	cry-COI-405F	CGTTACTCTTATTGAGCAGAT	none	405
	C. lupicaris L2	lup2-COI-467F	TCCTGATATAGCTTTTCCC	none	467
D	*C. newsteadi* N2	new2-COI-139F [3]	CTCCCAGTTCTTGCTGGT	none	139
	C. newsteadi s.s.	new-COI-231F	TTATTAATATGCGATCCGCC	none	231
	C. newsteadi N3	new3-COI-296F [3]	CATCTTCTCCCTACACCTG	none	296
	C. newsteadi N1	new1-COI-351F	TATATCCGCCTCTTTCAAGA	none	351
	C. selandicus	sel-COI-403F	TGACTATTATTAAGTAGCTTGGTA	T > G (23)	403

[1] For easier demarcation from *C. lupicaris* haplotype L2, *C. lupicaris* s.s. is designated as *C. lupicaris* haplotype L1 according to the nomenclature of Ander et al. [46]. [2] Modified primer from Nolan et al. [50]. [3] Modified primer from Pagès et al. [43].

To validate the specificity and sensitivity of the designed forward primers, the mPCRs were further tested with the DNA material of 41 genetically pre-identified specimens or the synthetic DNA of various taxa of the subgenus *Culicoides* (Table 2). All forward primers reliably detected their specific DNA, with the exception of the primer sel-COI-403F (mPCR D), which only generated specific PCR amplicons for two of three *C. selandicus* DNA samples, resulting in a total sensitivity of all mPCRs of 97.6%.

In terms of specificity, mPCRs A and B showed no unspecific annealing of the forward primers to non-target subgenus *Culicoides* taxa at all (100% specificity). However, three forward primers of mPCRs C and D showed weak signals with other subgenus *Culicoides* taxa: while using mPCR C, unspecific reaction signals were observed for del-COI-161F with the only *C. impunctatus* sample, for cry-COI-405F with one out of five tested *C. punctatus* samples and with *C. grisescens* haplotype G2, and for lup2-COI-467F with the synthetic DNA of *C. flavipulicaris*. In the case of mPCR D, the forward primer new3-COI-296F incorrectly reacted with the only *C. impunctatus* sample and with four of five DNA samples pre-identified as *C. lupicaris* haplotype L2. Additionally, one of five tested *C. punctatus* samples were identified as *C. newsteadi* haplotype N1 with the primer new1-COI-351F, and a 403 bp fragment of the genomic DNA of *C. grisescens* haplotype G2 (one sample tested) was amplified with the primer sel-COI-403F.

Finally, the mPCRs were tested with the genomic DNA of single biting midge specimens not belonging to the subgenus *Culicoides* (n = 21) but to other subgenera of the genus *Culicoides* to check whether the pre-sorting of biting midges to the group level is necessary before using the new PCRs. The agarose gel analyses summarized in Table 3 show no unspecific detection of tested *Culicoides* species with mPCR B. In the case of mPCR D, three unspecific DNA fragments with lengths of 120 bp, 550 bp and 900 bp were amplified when using the only *C. dewulfi* and *C. sanguisuga* DNAs as templates, but no unspecific signals occurred with the other 19 *Culicoides* taxa. mPCR A showed no unspecific amplicons at all, but the primer pun-COI-227F incorrectly detected *C. griseidorsum* and *C. pictipennis* as *C. punctatus*. Most cross-reactivity was observed for mPCR C: the primer lup1-COI-214F detected *C. riethi*, and the primer pul-COI-313F *C. poperinghensis* and the forward primer lup2-COI-467F amplified a 467 bp fragment with the genomic DNA of *C. festivipennis*, *C. kibunensis*, *C. obsoletus* clade O1 and *C. sanguisuga*. Additionally, one or more forward primers of mPCR C generated unspecific PCR amplicons with lengths of approximately 600 bp if *C. festivipennis* DNA was tested.

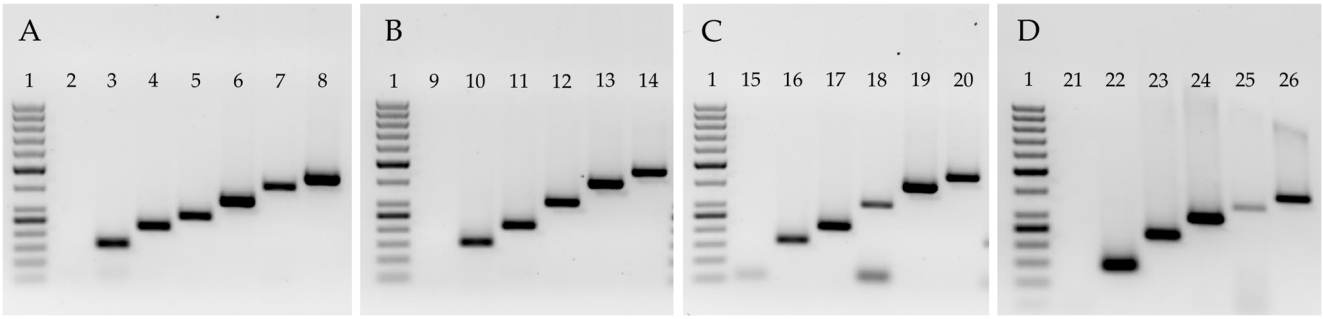

Figure 2. Validation of the different multiplex PCR tests (mPCRs (**A**–**D**)) for the subgenus *Culicoides* taxa. The specific primers used were as follows: bys-COI-158F, pun-COI-227F, boy-COI-275F, gri2-COI-346F, kal-COI-419F and gri1-COI-463F (lanes 2–8) for mPCR (**A**); fag2-COI-151F, fla-COI-215F, sub-COI-318F, fag1-COI-420F and imp-COI-491F (lanes 9–14) for mPCR (**B**); del-COI-161F, lup1-COI-214F, pul-COI-313F, cry-COI-405F and lup2-COI-467F (lanes 15–20) for mPCR (**C**); new2-COI-139F, new-COI-231F, new3-COI-296F, new1-COI-351F and sel-COI-403F (lanes 21–26) for mPCR (**D**). In all multiplex PCRs, forward primers were used in combination with the universal reverse primer PanCuli-COX1-727R. DNA samples used for PCR validation contained 10^6 synthetic COI gene copies or DNA extracts of single *Culicoides* specimens. Lane 1: 50 bp ladder (Gene Ruler, 50–1000 bp); lanes 2, 9, 15 and 21: no-template control; lane 3: *C. bysta*; lane 4: *C. punctatus*; lane 5: *C. boyi*; lane 6: *C. grisescens* haplotype G2; lane 7: *C. kalix*; lane 8: *C. grisescens* haplotype G1; lane 10: *C. fagineus* haplotype F2 (=*C. fagineus* s.s.); lane 11: *C. flavipulicaris*; lane 12: *C. subfagineus*; lane 13: *C. fagineus* haplotype F1; lane 14: *C. impunctatus*; lane 16: *C. delta*; lane 17: *C. lupicaris* haplotype L1; lane 18: *C. pulicaris* s.s.; lane 19: *C. cryptipulicaris*; lane 20: *C. lupicaris* haplotype L2; lane 22: *C. newsteadi* haplotype N2; lane 23: *C. newsteadi* s.s.; lane 24: *C. newsteadi* haplotype N3; lane 25: *C. newsteadi* haplotype N1; lane 26: *C. selandicus*. Primer-dimer formation was observed in the case of mPCR C (lanes 15 and 18).

Table 2. Results of specificity tests within the subgenus *Culicoides*. A total of 41 samples belonging to 21 taxa of the subgenus *Culicoides* were tested with the new multiplex PCRs, either with genomic DNA of single specimens or with 10^6 copies of specific synthetic COI gene DNA.

Species/Haplotype DNA	No. Samples	GenBank Accession No.	Multiplex PCR A						Multiplex PCR B						Multiplex PCR C						Multiplex PCR D				
			bys	pun	boy	G2	kal	G1	F2	fla	sub	F1	imp	del	L1	pul	cry	L2	N2	new	N3	N1	sel
C. bysta [1]	1	n.a	+ (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. punctatus [2]	5	OQ789061-65	-	+ (5)	-	-	-	-	-	-	-	-	-	-	-	-	+ (1)	-	-	-	-	+ (1)	-
C. boyi [1]	1	n.a	-	-	+ (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. grisescens G2 [2]	1	OQ789038	-	-	-	+ (1)	-	-	-	-	-	-	-	-	-	-	+ (1)	-	-	-	-	-	+ (1)
C. kalix [1]	1	n.a.	-	-	-	-	+ (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. grisescens G1 [2]	1	OQ789037	-	-	-	-	-	+ (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. fagineus F2 [2]	1	OQ789036	-	-	-	-	-	-	+ (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. flavipulicaris [1]	1	n.a.	-	-	-	-	-	-	-	+ (1)	-	-	-	-	-	-	-	+ (1)	-	-	-	-	-
C. subfagineus [1]	1	n.a.	-	-	-	-	-	-	-	-	+ (1)	-	-	-	-	-	-	-	-	-	-	-	-
C. fagineus F1 [1]	1	n.a.	-	-	-	-	-	-	-	-	-	+ (1)	-	-	-	-	-	-	-	-	-	-	-
C. impunctatus [2]	1	OQ789034	-	-	-	-	-	-	-	-	-	-	+ (1)	+ (1)	-	-	-	-	-	-	+ (1)	-	-
C. delta [2]	1	OQ789035	-	-	-	-	-	-	-	-	-	-	-	+ (1)	-	-	-	-	-	-	-	-	-
C. lupicaris L1 [2]	1	OQ789039	-	-	-	-	-	-	-	-	-	-	-	-	+ (1)	-	-	-	-	-	-	-	-
C. pulicaris s.s. [2]	6	OQ789055-60	-	-	-	-	-	-	-	-	-	-	-	-	-	+ (6)	-	-	-	-	-	-	-
C. cryptipulicaris [1]	1	n.a.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+ (1)	-	-	-	-	-	-
C. lupicaris L2 [2]	5	OQ789040-44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+ (5)	-	-	+ (4)	-	-
C. newsteadi N2 [1]	1	n.a.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+ (1)	-	-	-	-
C. newsteadi s.s. [1]	1	n.a.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+ (1)	-	-	-
C. newsteadi N3 [2]	4	OQ789048-51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+ (4)	-	-
C. newsteadi N1 [2]	3	OQ789045-47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+ (3)	-
C. selandicus [2]	3	OQ789052-54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+ (2)

+: Amplification; -: no amplification; (n): number of samples with amplification; n.a.: not applicable. [1] Synthetic COI gene DNA. [2] Genomic DNA of single specimens.

Table 3. Results of specificity tests with the four different multiplex PCRs with 21 species of the genus *Culicoides* not belonging to the subgenus *Culicoides*. One specimen of each species or haplotype was tested.

Species/Haplotype DNA	GenBank Accession No.	Multiplex PCR A					Multiplex PCR B						Multiplex PCR C				Multiplex PCR D				
		bys	**pun**	**boy**	**G2**	**kal**	**G1**	**F2**	**fla**	**sub**	**F1**	**imp**	**del**	**L1**	**pul**	**cry**	**L2**	**N2**	**new**	**N3**	**N1**	**sel**
C. achrayi	OQ789066	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. alazanicus	OQ789067	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. chiopterus	OQ789068	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. dewulfi	OQ789069	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+ [2,3]	+ [2,3]	+ [2,3]	+ [2,3]	+ [2,3]
C. festivipennis	OQ789070	-	-	-	-	-	-	-	-	-	-	-	+ [1]	+ [1]	+ [1]	+ [1]	+ [1]	-	-	-	-	-
C. griseidorsum	OQ789071	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. imicola	OQ789072	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. kibunensis	OQ789073	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
C. montanus	OQ789074	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. obsoletus clade O1	OQ789075	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
C. obsoletus clade O2	OQ789076	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. obsoletus clade O3	OQ789077	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. pallidicornis	OQ789078	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. pictipennis	OQ789079	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. poperinghensis	OQ789080	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C. riethi	OQ789081	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
C. riouxi	OQ789082	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. salinarius	OQ789083	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. sanguisuga	MK760238	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+ [4]	+ [4]	+ [4]	+ [4]	+ [4]
C. scoticus clade 2	OQ789084	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. sinanoensis	MK760244	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+: Amplification; -: no amplification. Unspecific amplicons with lengths of approx. [1] 600 bp, [2] 550 bp, [3] 900 bp and [4] 120 bp.

4. Discussion

In the past, several PCR tests were developed using either the mitochondrial COI gene [34,43,45,50,56,60] or nuclear-encoded ribosomal ITS1 [61–65] and ITS2 genes [65–67] to identify the species of the Obsoletus and Pulicaris Groups, which contain the putative biting midge vectors of BTV and SBV. GenBank DNA sequence analyses performed in the framework of the present study demonstrated that only the COI gene provides a sufficient number of entries for species and haplotypes of the subgenus *Culicoides* (COI: 495 sequences; ITS1: 15 sequences; ITS2: 39 sequences; as of September 2022), allowing the design of specific primers.

While most existent PCR assays for indigenous biting midge species are based on a limited number of COI sequences from field-collected specimens caught in a certain country or region, the present approach made use of all available sequence data for 21 subgenus *Culicoides* taxa deposited in GenBank. However, searching for suitable DNA sequences was not trivial with regard to the species variety within the subgenus, the wide use of synonyms for one and the same species [27] and misidentifications, which increase the chance of incorrect entries in databases. In this study, a significant number of GenBank COI sequences of subgenus *Culicoides* taxa were found to be incorrectly assigned to species (Table S1), which confirmed the results of previous studies [46,51,52] demonstrating the limitations of biting midge classification based on genetic data alone. Instead, it highlights the importance of the morphological definition of species and taxa whose sequences are to be entered into databases by experienced *Culicoides* taxonomists. It should also be discussed whether taxonomic changes have to be updated in such data repositories.

Despite these problems and the general issue of the genetic delimitation of species [68], a procedure was found to generate consensus sequences for the subgenus *Culicoides* taxa with high intraspecific homology. Further comparison of consensus sequences revealed variations between taxa, which suggests that some described cryptic taxa, especially those of *C. newsteadi* and the synonymous *C. delta* and *C. lupicaris*, should be regarded as separate species rather than genetic variants. This observation confirms the results obtained by Yildirim et al. [48]. The genetic variations were deployed for the development of specific primers, using identical cycling conditions for simultaneous sample analysis. The concept is based on the use of specific forward primers in combination with a universal reverse primer (PanCuli-COX1-727R) in a cost-effective and easy-to-use single-tube (multiplex) approach, generating one characteristic band for each taxon after the gel electrophoresis of PCR products. Another advantage of the idea of using one and the same reverse primer for all possible target DNAs is the efficient use of the COI gene fragment available in GenBank [50,56,69]. Its limited length of usually less than 500 base pairs reduces the options for primer positioning and makes the development of conventional multiplex PCR tests extremely difficult.

Despite many advantages, the application of a universal reverse primer simultaneously implies a great challenge: the specificity of the PCR is exclusively provided by the forward primer. Thus, in most cases, initial experiments resulted in the cross-reactive binding of potential primers to other taxa of the subgenus *Culicoides* and required more intensive testing. This issue could be solved by inserting single mismatch bases into the conserved regions of the primer sequence, assuming that mismatch base pairing would be less detrimental to the detection of the target taxa, according to the higher binding strength of the primer, than to non-target taxa. There was an attempt to apply the 'general hierarchy of mismatch impact' described in the literature [70–76]. However, except for the observation that incorrect base pairing at the 3′-terminal part of the primer should be avoided, it seemed to be more trial-and-error to find the best working mismatch in this study, which is not unexpected since many factors can influence the mismatch behavior [74,76]. After a considerable optimization process, primer specificity was adapted in a way that allowed the different taxa of the subgenus *Culicoides* to be distinguished, although some of the primers containing mismatches showed weaker binding strength than others. Thus, we elongated the affected primers despite the risk of reduced specificity, resulting in four functional

mPCRs detecting five to six species or haplotypes, each in a single reaction mixture. The sensitivity and specificity of these multiplex PCRs were checked as extensively as possible, although sample materials of species or haplotypes from the field were restricted. Among other things, this dilemma is caused by the comparatively low abundance of biting midges of the subgenus *Culicoides* as compared to those of the subgenus *Avaritia* (e.g., Obsoletus Group) in general and the over-representation of *C. pulicaris* and *C. punctatus* specimens within the subgenus *Culicoides* in particular in field collections from Germany [77–80]. In addition, in some taxa, only a few specimens have been found so far, probably due to the limited knowledge of their specific ecological niches and difficulties in identifying them. Missing materials for certain taxa were compensated for by using synthetic COI DNA.

In this study, PCR tests on four recently described species of the Pulicaris Group (*C. boyi*, *C. bysta*, *C. kalix* and *C. selandicus*) were developed for the first time, and all 21 newly designed specific forward primers for the subgenus *Culicoides* were able to identify their target taxa. In addition, 15 of the 21 forward primers showed no cross-reactivity with other members of the group if total DNA from single biting midges or equivalent (10^6 copies of synthetic COI gene) were tested, while six primers showed cross-talk within the subgenus *Culicoides* without a comprehensible explanation for how these primers were able to anneal to unspecific targets, especially if the number of mismatches (c.f. Table S4), the high annealing temperature and the use of hot-start Taq polymerase to reduce unspecific annealing are considered [57,58]. In one case, the forward primer for *C. delta* (del-COI-161F, mPCR C) produced a weak signal with the genomic DNA of the only *C. impunctatus* specimen available, although the primer sequence differed in 8 of the 23 bases, basically at the 3′-terminal part of the primer, from the sequence of the tested sample. Usually, such mismatch values prevent primer binding to unintended targets, and mismatches toward the 3′-end particularly hamper primer annealing [59]. Similar implausible results were obtained with the cry-COI-405F primer (mPCR C), incorrectly detecting one out of five *C. punctatus* samples (seven-base difference between primer and template sequence, with all tested samples having identical sequences) and the primer new3-COI-296F (mPCR D), incorrectly detecting four out of five *C. lupicaris* haplotype L2 samples (again, all with the same primer binding site sequence) despite mismatch pairing at seven positions. In another case, the primer (sel-COI-403F) only detected two out of three *C. selandicus* DNA samples despite the 100% sequence identity of all three specimens in the primer binding region. This false-negative result was attributed to the low DNA quantity of the non-identified sample. The DNA extraction of that one was performed with the NucleoMag VET Kit and a 100 µL elution volume, whereas the other two samples were processed with the QIAamp DNA Mini Kit and eluted in 50 µL of buffer. In the case of the only available *C. impunctatus* sample, which was non-specifically detected with the *C. delta*- and *C. newsteadi* haplotype N3-specific forward primers, a DNA extract from a previous study was used, which cannot be excluded without a doubt to have originated from a pool of biting midges. In order to circumvent such uncertainties, each DNA sample was generally sequenced before use; however, it might be possible that only the more abundant species within a mixed pool was determined. With respect to the other cross-reactions observed within the subgenus *Culicoides*, a plausible explanation cannot be found yet. Despite all preventive measures to avoid contamination and methodological measures to avoid unspecific annealing, as well as the application of the 'four eyes' principle during sample preparation, individual mistakes, including the confusion of tubes, cannot be ruled out.

Unspecific binding was also observed in several cases in which biting midge species not belonging to the subgenus *Culicoides* were tested. For instance, although in the primer pun-COI-227F (mPCR A), there are exchanges of two bases compared to *C. griseidorsum* DNA and three bases compared to *C. pictipennis* DNA, all of them exclusively in the middle and at the 5′-terminal part of the primer (Table S4), unspecific binding occurred with the DNA of these species. This is not unusual, as only a few mismatches in the middle or at the 5′-end of the primer do not necessarily lead to the complete loss of primer binding

capacity, which is exploited, for example, in site-directed mutagenesis or the insertion of restriction sites [59]. However, the PCRs were meant to differentiate taxa within the subgenus *Culicoides*, and cross-reactivity with taxa not belonging to this subgenus appears to be extremely difficult to avoid, if not impossible, simply because of the huge number of taxa to be considered. These cases in fact demonstrate the importance of the morphological pre-sorting of biting midges to the group level before genetic examination.

Unfortunately, morphological pre-sorting is time-consuming and unsuitable for the high-throughput approaches needed to process the tremendous numbers of biting midges usually obtained from field collections. According to this, and considering that classical taxonomists are becoming an 'extinct species', there is a great need for finding alternative techniques for *Culicoides* classification. A biochemical method for species-specific protein profiling, Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF), was suggested to soon become a reference method for arthropod identification [81], as it is rapid and cost-effective and does not require entomological expertise or special training [81]. Subsequently, several authors successfully used this method for the species identification of adult [82–86] and juvenile *Culicoides* specimens [87]. However, like all methods, MALDI-TOF has some disadvantages: (i) comparatively high costs for initial equipment [88,89], (ii) results do not provide evolutionary information for phylogenetic analyses [39], (iii) incongruent protein spectra, depending on the developmental stage of the insect [81,87] and the selected body parts used for analysis [81], (iv) no public library available with a sufficient number of reference protein profiles of each *Culicoides* species or haplotype and, finally, (v) relatively low resolution and limited sensitivity, insufficient to distinguish very closely related species [81]. Facing these obstacles, scientists need to think of more creative ways for the fast and accurate classification of *Culicoides*. Perhaps, artificial intelligence will help develop new identification methods in the future.

So far, PCR-based approaches seem to be the methods of choice, and in particular, quantitative real-time PCR is becoming increasingly important because of the possibility of analyzing pooled specimens [39]. Since there are indications that variations in the COI gene are insufficient for species delimitation within some subgenera [46,90], which could be confirmed by the present work, the implementation of multi-marker PCR approaches might be a great advantage, as already demonstrated in a recent study using several gene loci for phylogenetic analysis within the subgenus *Avaritia* [91].

Our results clearly demonstrate that developing multiplex PCR tests is a great challenge, merely based on the number of molecules used together in one reaction mixture and the multitude of possible interactions between them. Due to restricted availability, the newly developed tests were evaluated with a limited number of specimens and need further evaluation with additional samples, including pools of subgenus *Culicoides* biting midges, as tested during PCR development for the Obsoletus Group in a previous study [65]. However, the mPCR tests described here enable the parallel identification of almost all taxa of the subgenus *Culicoides* for the first time, among them recently described genetic variants and species not detectable with published PCR tests.

Moreover, it was attempted to keep the PCRs as simple as possible: in contrast to the PCR test of Pagès et al. [43], the utilization of one specific forward primer per species or haplotype, in combination with a universal reverse primer, decreases costs and simplifies the PCR evaluation. As opposed to previous PCRs developed for the Pulicaris Group and its relatives [43,50], the annealing temperature of the new mPCRs was generalized, thus applying a uniform PCR temperature profile and simplifying the experimental protocol. Depending on the aim of the study, each specific forward primer can also be used together with the universal reverse primer in a singleplex approach, which again reduces the cost per reaction. Each of the four mPCRs can be performed with fewer primers than suggested, but specific primers should not be mixed in other combinations in order to reduce primer-dimer formation and avoid the simultaneous production of amplicons indistinguishable by length. Despite the observed unspecific binding of individual primers, the first results with the PCRs are promising and indicate the great potential of our tests to improve the

identification of suspected vector species within the subgenus *Culicoides* and the knowledge on biting midge distribution and ecology.

5. Conclusions

The results presented in this study confirm the great potential of the COI marker for species identification within the culicoid subgenus *Culicoides* [28,46]. The aim of the study, the development of PCR tests for the differentiation of species and haplotypes of the subgenus *Culicoides*, and the members of the Pulicaris Group in particular, was achieved through bioinformatic analysis of all COI sequences available from GenBank. This successful approach stresses the importance of such databases and resulted in different multiplex assays now becoming available to identify taxa of the subgenus *Culicoides*. A particular achievement of the assays is the inclusion of recently discovered species and haplotypes, for which no PCR identification tests have been available so far and whose ecologies and vector roles are completely unknown. Nonetheless, further testing with more specimens from field collections has to be performed to confirm the reproducibility and the benefit of the developed tests. Future analysis of the complete mitochondrial genome of *Culicoides* could significantly increase the possibilities of genetic differentiation and help unveil systematic issues.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d15060699/s1. Table S1: GenBank entries and consensus sequences used for the development of forward primers specific for the different taxa of the subgenus *Culicoides*; Table S2: Sequences of synthetic COI genes of subgenus *Culicoides* taxa used in this study; Table S3: List of all forward primers tested in this study; Table S4: Cross-talk of newly designed primers with the taxa of the subgenus *Culicoides* and of culicoid genera other than *Culicoides*.

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Data Availability Statement: Publicly available datasets were analyzed in this study and are listed in the Supplementary Material (Table S1). Respective data can be found in GenBank at: https://www.ncbi.nlm.nih.gov/genbank/ (accessed on 28 October 2022). New data created in this study are deposited in the same data repository with the following accession numbers: *C. impunctatus*: OQ789034; *C. delta*: OQ789035; *C. fagineus* haplotype F2: OQ789036; *C. grisescens* haplotype G1: OQ789037; *C. grisescens* haplotype G2: OQ789038; *C. lupicaris* haplotype L1: OQ789039; *C. lupicaris* haplotype L2: OQ789040-OQ789044; *C. newsteadi* haplotype N1: OQ789045-OQ789047; *C. newsteadi* haplotype N3: OQ789048-OQ789051; *C. selandicus*: OQ789052-OQ789054; *C. pulicaris*: OQ789055-OQ789060; *C. punctatus*: OQ789061-OQ789065; *C. achrayi*: OQ789066; *C. alazanicus*: OQ789067; *C. chiopterus*: OQ789068; *C. dewulfi*: OQ789069; *C. festivipennis*: OQ789070; *C. griseidorsum*: OQ789071; *C. imicola*: OQ789072; *C. kibunensis*: OQ789073; *C. montanus*: OQ789074; *C. obsoletus* clade O1: OQ789075; *C. obsoletus* clade O2: OQ789076; *C. obsoletus* clade O3: OQ789077; *C. pallidicornis*: OQ789078; *C. pictipennis*: OQ789079; *C. poperinghensis*: OQ789080; *C. riethi*: OQ789081; *C. riouxi*: OQ789082; *C. salinarius*: OQ789083; *C. scoticus* clade 2: OQ789084.

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