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Optimization of Primer Candidate Design for *Toxocara cati* Identification Using PCR Targeting the *COX1* and *ND5* Genes in Cats

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Abstract

Toxocara cati is an obligate extracellular parasite within the phylum Nematoda. This species is responsible for toxocariasis, a zoonotic disease. The disease transmission occurs via infective eggs, earthworms, cockroaches, birds, and rodents that contain larvae in their tissues. In cats, infection with this parasite can lead to symptoms such as diarrhea, hypoalbuminemia, anorexia, and a distended abdomen. This research aimed to identify an optimal candidate primer design through *in silico* analysis using BLAST primers from the National Center for Biotechnology Information (NCBI). This study targeted the mitochondrial genes *COX1* and *ND5* for the molecular identification of *T. cati*. Using *in silico* methods, primer candidates were designed and evaluated based on key parameters, including primer length, melting temperature (Tm), GC content, potential for secondary structure formation, and specificity. Candidate primers were screened using the NCBI primer-BLAST tool and validated through BLAST analysis to ensure sequence specificity. The primer pair that m*et a*ll the criteria for an optimal candidate primer design comprised the forward primer TC*ND5*F (5′-ACTGCTGGCCTTGTATTGGT-3′) and the reverse primer TC*ND5*R (5′-ACACAGAACGCCTAAACCTCA-3′), both targeting the *ND5* gene region.

Keywords: *COX1* gene, *ND5* gene, PCR, *Toxocara cati*

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INTRODUCTION

Toxocariasis is a zoonotic disease caused by the larvae of *Toxocara*, a genus of roundworms from the ascaroid group, with a worldwide distribution. In cats, the species of primary concern is *T. cati*. Infected cats may exhibit symptoms such as weight loss, dull coat, abdominal distension, vomiting, and diarrhea. Larval migration can affect the respiratory system, potentially leading to pneumonia. These worms impair nutrient absorption, causing emaciation, and in severe cases, can be fatal, posing significant public health concerns (Murniati *et al*., 2016). The prevalence of *T. cati* in cats across Indonesia varied: 23.9% in Banyuwangi, 11% in Padang, 35% in Bogor, 16.6% in Banjarnegara, 65% in Bali, and 60.9% in Surabaya (Suroiyah *et al*., 2018). Humans can become infected either by consuming meat or organs containing infective larvae or by ingesting embryonated eggs of these parasites (Zhou *et al*., 2023).

In humans, toxocariasis manifests in four clinical forms: visceral larva migrans, ocular larva migrans, covert toxocariasis, and neurological toxocariasis. Toxocariasis is a zoonotic infection caused by the larval stages of *Toxocara* spp., capable of inducing both systemic and ocular manifestations in humans (Khademvatan *et al*., 2013). Although early clinical documentation of human toxocariasis in Indonesia is limited, several case reports and seroepidemiological studies have confirmed the endemic presence of this parasite. For instance, Gunawan and Zulfan (2024) reported a rare case of Wells' syndrome triggered by co-infection with *Toxocara* and *Strongyloides* in an Indonesian patient, emphasizing the immunopathological complexity of the disease. Furthermore, a serological survey conducted in North Sulawesi found a high prevalence of

Toxocara spp. antibodies in junior high school students, with 84.6% testing positive despite being asymptomatic. These findings suggest that toxocariasis represents a significant public health issue in Indonesia, particularly among children who are more vulnerable due to environmental exposure and behavioral factors such as geophagia. *Toxocara* spp. infection in humans was first described within a child's retinal granuloma. Since then, larvae of these *Toxocara* species have been found in a range of ocular and systemic diseases in patients worldwide. Toxocariasis, in all its clinical forms, is now recognized as a parasitic disease of public health concern which imposes a significant health risk, particularly for children exhibiting pica behavior (Ma *et al*., 2018; Despommier, 2003; Rostami *et al*., 2019). Pica behavior is an eating disorder involving recurrent intake of non-food substances (e.g., paper, soil) (Papina *et al.*, 2024), which is strongly associated with an increased risk of ingesting *Toxocara* eggs. These eggs require several weeks to embryonate in the environment, making contaminated sandboxes and poorly maintained yards potential sources of infection due to poor pet hygiene (Maraghi *et al*., 2014).

Traditionally, diagnosis of toxocariasis in cats involves fecal microscopic tests, general fecal examination, physical examination, and assessment of clinical symptoms. However, general fecal examination methods often lack sensitivity, especially when the quantities of feces and parasites are insufficient (Elsemore *et al*., 2023). Additionally, the presence of debris in the feces can complicate diagnosis. The flotation method has limitations due to sugar solution crystallization, which obscures microscopic visibility, the failure to detect heavier parasite eggs, which leads to false negatives, and the intermittent shedding of parasites, which requires repeated sampling (Englar and Bessett, 2021). Consequently, molecular testing to identify specific parasite DNA in feces offers the most effective approach. Detection methods with high sensitivity and specificity, such as polymerase chain reaction (PCR), provide accurate results because they can identify parasitic DNA even in samples with low parasite burdens, ensuring early and accurate diagnosis. Polymerase chain reaction allows specific identification of another species, which is difficult to achieve with microscopic examination due to similarity in shape to *T. cati* (Khademvatan *et al.*, 2013).

PCR is utilized for specific diagnosis of toxocariasis because it can selectively amplify DNA from adult worms, larvae, and eggs. Effective primer design is essential for accurate parasite identification through PCR (Khademvatan *et al*., 2013). Primers act as markers for the DNA fragments to be amplified, and optimal primer design ensures alignment with the target sequence, maximizing the correct target sequence's amplification in the parasite genome (Dewi *et al*., 2019). Successful DNA amplification in PCR depends on the primers specifically designed for the target sequence. *In silico* tools are used to design candidate primers (Purwakasih and Achyar, 2021). Ideal primers should be 18–30 nucleotides long and have a GC (Guanine–Cytosine) content of 40–60%, and the forward and reverse primers should have a melting temperature (Tm) difference of no more than 5°C, while avoiding secondary structures like hairpins or self-dimers. *In silico* analysis is crucial for designing primers, which must be validated in the laboratory through optimization of annealing temperature (Ta) and primer concentration (Saraswati *et al*., 2019). The design of accurate primers for PCR-based diagnosis of toxocariasis requires further study. While *T. cati* DNA shows genetic variation across regions, no primers have been developed for isolates from Indonesia, particularly Malang. This highlights the urgent need for locally adapted primers to improve detection and monitoring in endemic areas.

This study aimed to develop *in-silico*-designed primers from protein coding and mitochondrial genes for effective and efficient use in the PCR amplification process. This study is essential for creating a potential primary product for parasite identification that is applicable in research laboratories in Indonesia and globally. The innovation from this study could enhance the accuracy of parasitic diseases identification, including those of public health

significance, and thus improve the treatment and prevention of zoonotic diseases. We focused on optimizing conventional PCR by targeting the *COX1* and *ND5* genes. Low-cost molecular techniques using a single stool sample can overcome the limitations of microscopy in detecting infections. This highly sensitive method can detect parasite DNA concentrations as low as 0.001 ng/mL from a single fecal sample, offering a cost-effective and scalable solution.

Several previous studies have employed PCR to detect *T. cati* by targeting nuclear or mitochondrial markers such as the *ITS* gene (Khademvatan *et al*., 2013) or the *nad1* gene from intestinal content to detect the presence of adult worms (El-Seify *et al*., 2021). However, no research to date has applied the *ND5* gene for *T. cati* detection. Although *COX1* has been widely used in global phylogenetic studies due to its high mutation rate and suitability for DNA barcoding, it has yet to be employed in molecular detection of *T. cati* in Indonesia. The use of mitochondrial genes such as *COX1* and *ND5* offers practical advantages, including a higher copy number per cell, simplifying DNA isolation. Moreover, *COX1* is preferred for its low rate of insertions and deletions, while *ND5*, a subunit of the NADH dehydrogenase complex in the inner mitochondrial membrane, provides valuable resolution for assessing genetic and phylogenetic diversity among closely related species. Taken together, this study provides a critical step toward establishing a reliable and robust primer-based diagnostic platform for *T. cati*, with promising implications for advancing molecular parasitology and veterinary diagnostics in endemic regions.

MATERIALS AND METHODS

Ethical Approval

The Animal Care Committee at Universitas Brawijaya approved the animal ethics protocol with reference number 065-KEP-UB-2023.

Study Period and Location

This study was conducted from April to October 2024, with sample collection carried out in the Malang area, followed by collection and morphological identification at the Laboratory of Parasitology, Faculty of Veterinary Medicine, Universitas Brawijaya, and molecular identification and primer optimization performed at the Animal Disease Diagnostic (ADD) Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya.

Samples and Microscopical Examination

T. cati eggs were initially identified by visual analysis of fresh fecal samples from three cats from an animal shelter in Malang, using a modified flotation technique as described by Cringoli *et al*. (2010). Two grams of feces were processed with 18 milliliters of sucrose solution (specific gravity of 1.27) and subjected to centrifugation. The floating material was subsequently transferred to a slide for microscopic examination (Olympus CX-23, Japan), followed by the identification of the morphological characteristics of *T. cati* eggs, including their round or pear-shaped, thick pitted shell and brownish coloration.

Adult Worm Sample Collection

A total of three adult *T. cati* worms were obtained from naturally infected cats at an animal shelter in Malang, Indonesia, following anthelmintic administration (Drontal Cat®), which contains the active ingredients Praziquantel and Pyrantel Embonate. The collection criteria included the presence of white roundworms and prior confirmation of *T. cati* infection through fecal examination. For a molecular study, the worms were immediately preserved in absolute ethanol for DNA extraction (Sepulveda and Kinsella, 2013).

DNA Isolation

The DNA isolation process began with subjecting adult *T. cati* worms to evaporation from ethanol at room temperature for two days, which caused them to dry up. The worms were then cut into 0.5-cm pieces and placed into microtubes for DNA extraction. Each tube was treated with 200 microliters of nuclei lysis solution and 60 microliters of Proteinase K, a

broad-range protease that releases nucleic acids and breaks down cell proteins (Qamar *et al*., 2017). The microtubes were sealed with Parafilm® and incubated at 55°C for 16–18 hours. This procedure utilized the Wizard® SV Genomic DNA Purification System, developed and manufactured by Promega Corporation in the USA, including all necessary reagents for genomic DNA isolation such as nuclei lysis solution, EDTA, Proteinase K, and wash solution, as outlined in the purification protocol.

In Silico Primer Design

The selection of target genes was based on their base pair lengths. Nucleotide sequences of the *COX1* and *ND5* genes were copied from the FASTA section and analyzed for candidate primer selection using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The specificity of the selected primers was assessed using the BLAST feature at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), with parameters set to 100% query cover, 100% identity, and low E-values. BLAST results guided the design of primers targeting specific gene regions.

In Vitro DNA Amplification

The PCR method used a Bio-Rad® T100™ Thermal Cycler manufactured in Singapore and involved several stages: pre-denaturation, denaturation, annealing, elongation, post-elongation, and cooling. DNA isolates from three *T. cati* worm samples in Malang were used. The PCR mixture included 1 µL of forward primer, 1 µL of reverse primer, 6 µL of MyTaq HS Red Mix 2X PCR Master Mix, 1 µL of ddH_2O or nuclease-free water, and 3 µL of DNA isolate. The samples were homogenized using a Velp® vortex mixer manufactured in VELP Scientifica Srl in Italy and then processed in the thermal cycler.

Sanger Sequencing

PCR products (45 µL) as well as forward and reverse primers (30 µL each) were coded and sealed with Parafilm, then packed in Styrofoam boxes for shipment to PT. Genetika Science Indonesia and subsequently to 1st Base Malaysia for sequencing.

In Silico PCR Analysis

PCR analysis was conducted using MEGA Version 11.0. The best primers were identified based on electrophoresis results showing clear and distinct bands. The *T. cati* DNA sequence from the NCBI GenBank site was aligned with the forward primer using the Clustal W algorithm. The reverse primer was aligned via reverse complementation and then linked to the *T. cati* sequence. This alignment was also done using the Clustal W algorithm. Sequence alignment results were analyzed using BLAST and phylogenetic tools. The phylogenetic tree was reconstructed using the Maximum Likelihood (ML) method with the Kimura 2-parameter model and a 1000-bootstrap test. The use of Maximum Likelihood with the Kimura 2-parameter ensured that the phylogenetic tree was both statistically supported and meaningful, accurately reflecting genetic similarities and differences between *T. cati* and closely related species.

Data Analysis

Electrophoresis results of the candidate primer designs were analyzed descriptively. Bands were scored based on thickness and number, following criteria from Seeker *et al*. (2016). Scores were averaged, with the lowest average indicating the best primer design for *T. cati*.

RESULTS AND DISCUSSION

In Silico Primer Design Results

The *T. cati* DNA sequence was obtained from the GenBank database at http://www.ncbi.nlm.nih.gov/, using one complete mitochondrial genome with accession number NC_010773.1 in FASTA format, with a nucleotide length of 14,029 bp. Template selection was based on the base length analysis of each gene. This sequence was selected because it provided the full-length nucleotide data required for *in silico* primer design, including the target regions of the *COX1* and *ND5* genes. According

to Nishio *et al*. (2021), longer templates tend to have better gene expression levels. The *COX1* gene was located between positions 6,055 and 7,632, with a total length of 1,577 bp, while the *ND5* gene was between positions 9,926 and 11,507, with a total length of 1,581 bp.

Three primer pairs generated via NCBI Primer-BLAST were ranked based on optimal criteria, including ideal length (18–30 bp), Tm difference ≤5°C, GC content at 40–60%, and minimal self- and 3′-complementarity. The results of electrophoresis and analysis of the primer design candidates are summarized in Figure 1 and Table 1.

Sequencing Chromatogram Results

The chromatogram in the TC*ND5* primer design sequencing shows suboptimal quality, characterized by overlapping curves and imperfect peaks (Figure 2). Optimal chromatogram peaks are clear and do not overlap, which indicates high-quality sequencing (Taariwun *et al*. 2021). Suboptimal sequencing can result from the primer design's self-complementarity, leading to misalignment or errors in nucleotide sequencing (Al-Shuhaib and Hashim, 2023).

Table 1. Primer design of *T. cati*

Code		Primer design	Length of product	Tm (°C)	GC %	*Self-complementarity*	*Self-3' complementarity*
TC*COX1*A	F	TGTGCCTACGGGTGTTAAGG	550	59.68	55.00	4.00	0.00
	R	AGCCCTATACTCCGGCCTAC		60.25	60.00	4.00	2.00
TC*COX1*B	F	TTGTAGATATGGGGTGTGGG	594	56.29	50.00	4.00	0.00
	R	TAGGCACAGCGATAACCATA		56.12	45.00	2.00	1.00
TC*ND5*	R	ACTGCTGGCCTTGTATTGGT	553	59.59	50.00	4.00	0.00
	P	ACACAGAACGCCTAAACCTCA		59.59	47.62	2.00	1.00

Primers were evaluated using NCBI Primer-BLAST based on standard parameters including product length, melting temperature (TM), GC content, self complementary, and 3' complementarity.

Table 2. Electrophoresis scoring primer design of *T. cati*

Sample	Primer		
	TC*COX1*A	**TC*COX1*B**	**TC*ND5***
1	5	1	1
2	5	4	1
3	5	3	1
Total	15	8	3
$\underline{X}$	5	2.6	1

Scores represent visual assessment of PCR band quality, based on Seeker *et al*. (2016). A lower score indicates better band clarity, intensity, and specificity. The TC*ND5* primer pair, which consistently produced clear, single bands across all samples, obtained the lowest cumulative score and was selected as the optimal primer for further sequencing and phylogenetic analysis.

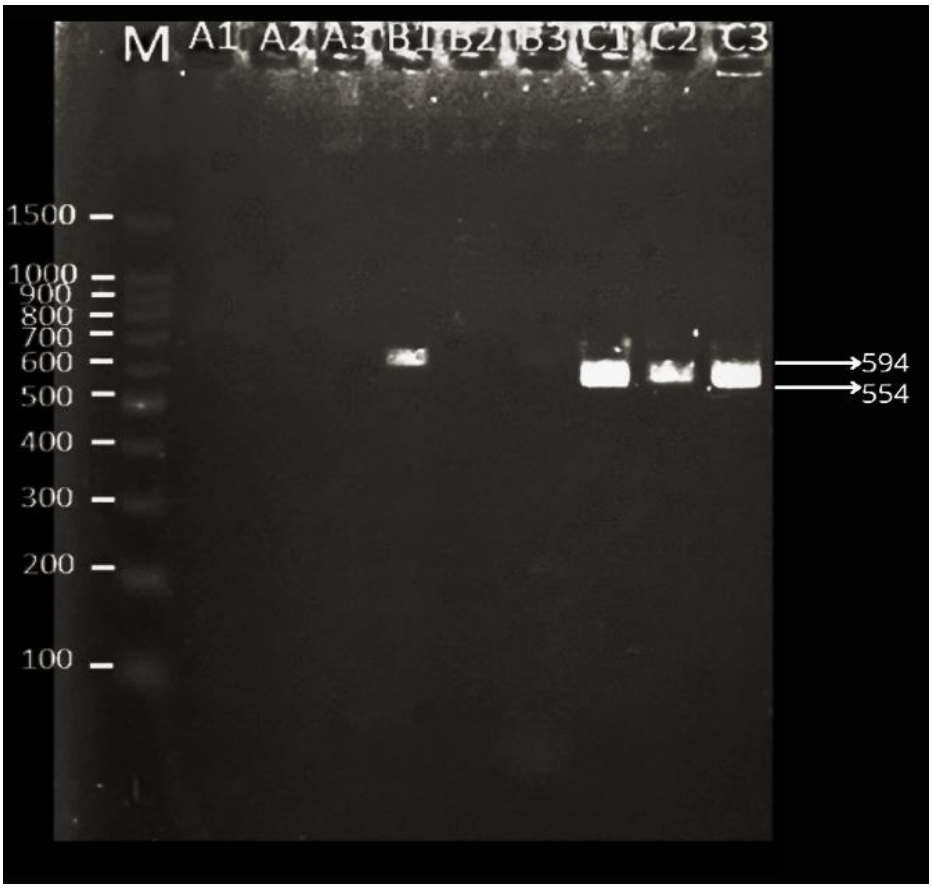

Figure 1. PCR Product of Primer Design Amplification for *T. cati.* (M) Marker, (Lanes 2–4) TC*COX1*A primer design, (Lanes 5–7) TC*COX1*B primer design, (Lanes 8–10) TC*ND5* primer design.

Figure 2. The chromatogram results in the TC*ND5* primer design sequencing. (A) reverse, (B) forward.

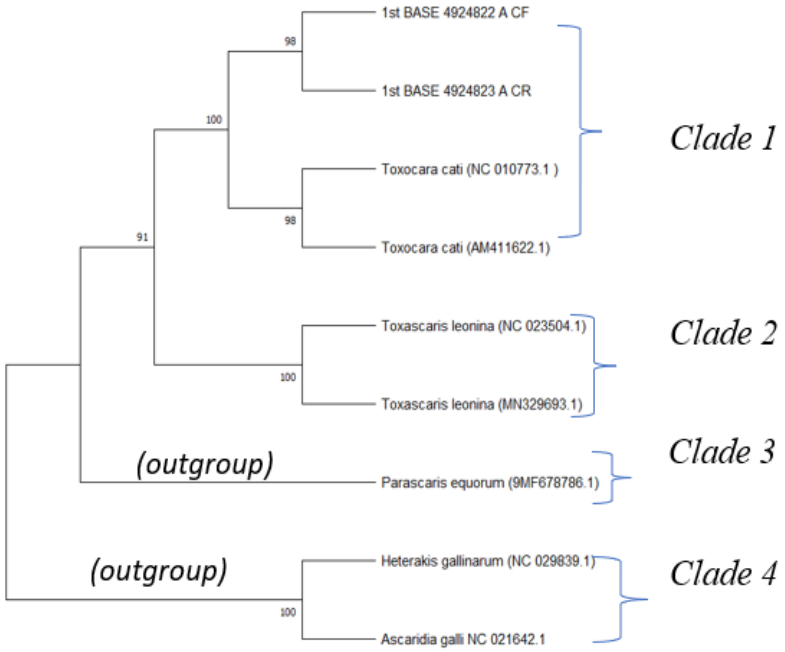

Figure 3. Phylogenetic tree reconstruction based on TC*ND5* sequencing results. The phylogenetic tree was constructed using *T. cati, Toxascaris leonina* and outgroup sequences retrieved from NCBI GenBank. The Maximum Likelihood method with the Kimura 2-parameter model and 100 Bootstrap tests were employed.

PCR Analysis Results for the *ND5* Gene of *T. cati* Using the *In Silico* Method

The *in silico* method was employed to analyze the sequencing results for *T. cati*, targeting the *ND5* gene. This analysis was conducted using the Molecular Evolutionary Genetic Analysis (MEGA) software, version 11.0. The primer chosen was the one with the best score as shown in Table 2. In this method, the reverse primer was matched with the forward primer using reverse complementation, followed by alignment using the Clustal W algorithm. The alignment results were tested with BLAST to assess the specificity of the primer design. The BLAST data showed the highest level of homology between *T. cati* and *Toxascaris leonina*. This strong sequence similarity was due to the close taxonomic relationship between these species, including class, phylum, and order, indicating a close evolutionary relationship. However, each species possesses unique genetic markers that differentiate them from related species or those with similar morphological characteristics. The E-value for *T. cati*, being close to 0, indicates a high level of sequence homology (Okulewicz *et al*., 2012).

The Phylogenetic Tree

The phylogeny of worms only clustered according to hosts but apparently not according to geography despite the assessment of worms from different continents (Taariwun *et al*., 2021). Clade 1, with a bootstrap value of 100%, comprised two subclades, the first of which included the sequencing results from the TC*ND5* primer design, with a bootstrap value of 98%, alongside *T. cati* sequences (NC_010773.1 and AM411622.1), both also with a bootstrap value of 98%. The second clade contained *T. leonina* (NC_023504.1 and MN329693.1) with a bootstrap value of 100%. The third clade featured *Parascaris equorum* as an outgroup with a bootstrap value of 91%. The fourth clade included *Heterakis gallinarum* and *Ascaridia galli*, both being outgroups, with a bootstrap value of 100% (Figure 3).

In this study, the three candidate primers met the standard criteria for primer eligibility, with lengths of 20–21 bases. According to Purwakasih and Achyar (2021), ideal primers have a nucleotide length of 18–30 bp. Short primers may cause mispriming and non-specific amplification, while excessively long primers do not improve

specificity but increase costs (Fava *et al*., 2020). Other optimal primer criteria concern the melting temperature (Tm) difference between forward and reverse primers and the overall Tm range. Tm influences the annealing temperature setting in PCR. For the TC*COX1*AF–TC*COX1*AR primer pair, the Tm difference was 0.57°C, for the TC*COX1*BF–TC*COX1*BR pair it was 0.17°C, and for the TC*ND5*F–TC*ND5*R pair there was no Tm difference. The Tm ranges for the three primer candidates were 56–60°C. These Tm differences and ranges comply with optimal primer standard criteria. According to Prakoso *et al*. (2016), the Tm difference should be minimal to prevent reduced amplification efficiency. Ideally, primer Tm should be between 42°C and 65°C; Tm values above 65°C can impair annealing efficiency, adversely affecting DNA amplification (Yustinadewi *et al*., 2018).

Regarding GC content, the three primer candidates ranged from 45% to 60%, meeting optimal design criteria. Pradnyaniti and Yowani (2013) highlighted that the ideal primer should have a GC% range of 40–60%. Lower GC% can reduce PCR efficiency due to weaker competition in template binding, thus hindering DNA amplification. For self-complementarity, lower values indicate a more ideal primer design. The maximum self-3′ complementarity should be 3, and the maximum repetitive bases should be 4. All three designs adhered to these criteria, indicating minimal self-annealing and higher efficiency in targeting desired DNA. High self-3′ complementarity can lead to primer self-binding, reducing amplification efficiency by interfering with proper attachment to the DNA target, thus hampering the PCR process (Benson *et al*., 2013).

The candidate primers were analyzed using Basic Local Alignment Search Tool (BLAST) to compare them against sequence databases, ensuring design specificity with the target sequences (Suparman *et al*., 2016; Firdausy *et al*., 2025). Ideal primer specificity is indicated by a query coverage close to 100%, an E-value near 0, and an identity (Ident) close to 100% in each database. Query coverage reflects the match percentage between the sample and NCBI database sequences. The E-value measures the statistical significance of matches found, while Ident denotes the sequence match level between the sample and the NCBI database (Benson *et al*., 2013).

The TC*COX1*A primer design did not yield DNA bands in any of the three samples (Table 2). The absence of bands may be attributed to suboptimal primer design factors. Despite optimization attempts at annealing temperatures of 53°C and 52°C, no DNA bands were produced. The TC*COX1*A primer exhibited a self-complementarity score of 4 in both forward and reverse primers, and a self-3′ complementarity score of 2 in the forward primer. According to Budiarto *et al*. (2019), amplification failures may occur due to secondary structure formation. Other factors contributing to amplification failure include mismatched concentrations between the primer and the DNA template, as well as insufficient DNA isolation concentration, which can impair PCR efficiency (Utaminingsih and Sophian, 2022).

The visualization results for the TC*COX1*B primer design showed variable and suboptimal band formation, with dimers in sample 3, and smears and multiple bands in sample 2. These imperfect DNA bands may result from the suboptimal primer design and PCR optimization processes. The multi bands observed in lane B2 might be due to a sequence repetition of four consecutive G bases in the forward primer of the TC*COX1*B design. While a sequence run of four bases is generally acceptable, it can sometimes lead to mispriming, causing primers to bind to unintended targets (Prakoso *et al*., 2016). Wulandari *et al*. (2017) noted that multiple bands formation can also occur if the annealing temperature is too low, leading to the creation of non-specific DNA bands. Smears may result from contaminants in the DNA preparation. The presence of dimers in lane B3 suggest primer–primer interactions forming short fragments, potentially caused by insufficient DNA sample concentration (Almeida *et al.*, 2011). Melati *et al*. (2019) indicated that primer dimer formation is likely when self-complementarity and self-3′ complementarity values are not zero.

The percent identity for *T. cati* is 99.08%, higher than that of *T. leonina*, demonstrating a greater match between the searched sequence and NCBI sequence data (Sihotang *et al*., 2021). Limited data on the GenBank can affect BLAST results, as GenBank entries come from individual laboratories or large-scale research inputs, leading to potential data limitations (Benson *et al*., 2013). The results were then re-aligned using the Clustal W algorithm, comparing them with the *ND5* gene sequence of *T. leonina* obtained from the NCBI GenBank. Outgroup species were selected from the Ascaridia order to validate the specificity of the primer design for *T. cati*. The chosen outgroups were *P. equorum* (accession number MF678786.1), *H. gallinarum* (NC_029839.1), and *A. galli* (NC_021642.1). The outgroups were selected based on their relatedness to the ingroup, but they should not as closely related as the ingroup members (Zhou *et al*., 2023).

Species diversification can be represented like a tree branching process, where each branch represents a species and is connected to other tree branches, showing relatedness (Subari *et al*., 2021). A phylogenetic tree which shows a bootstrap value above 70% is considered robust, while a lower value suggests that the dataset used for the model may be unreliable. This indicates that the primer design remained specific to *T. cati* even when compared against other species within the same order.

CONCLUSION

The optimal primer design for *T. cati* was achieved by optimizing criteria such as primer length (18–30 bases), GC% value (40–60), melting temperature difference (3–5°C), minimal secondary structure, and BLAST confirmation of specificity to *T. cati* using NCBI Genbank data. The optimized primer design for the *ND5* gene target (TC*ND5*) was proven to be more sensitive and reliable than *COX1*-based PCR primers for *T. cati*, yielding clean, clear, and smear-free bands, with a target size of 550 bp, and optimal performance was observed at a temperature of 53°C. Phylogenetic tree reconstruction confirmed the specificity of the primer design. However, the optimization of the primer design for the *COX1* gene target resulted in suboptimal outcomes, characterized by inappropriate band scoring, smear formation, and the presence of dimers. The TC*ND5* primers can be developed as a standard diagnostic tool for *T. cati* in veterinary and public health. They may be used for large-scale testing of clinical and environmental samples. Future studies should explore their ability to detect low-level infections and combine them with other primers for detecting multiple parasites at once.

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AUTHORS' CONTRIBUTIONS

The authors gratefully acknowledge the contributions of all team members. RY was responsible for developing the research ideas, conducting data analysis, preparing the manuscript, leading the investigation, and securing funding. NKN contributed to methodology, data analysis, manuscript preparation, and sampling. SKR provided support in data analysis. JS contributed through critical review and revision of the manuscript. All authors have read and approve the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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