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Specific PCR primers for rapid detection of five rat and mouse species in Java, Indonesia

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ABSTRACT Identifying rat and mouse species quickly, affordably, and accurately is crucial for effective population management, as well as for eradication or conservation purposes. However, the sheer diversity of these species poses a challenge. To address this, a molecular approach has been developed, involving the amplification of a short genetic marker from materials commonly left by the animal, such as hairs and feces. Recent available PCR primers were not suitable for the surveillance of large sample sizes. As a solution, this study designed and validated a PCR primer set capable of detecting five species of rats and mice (*Mus musculus*, *Rattus tanezumi*, *Bandicota indica*, *Rattus tiomanicus*, and *Rattus argentiventer*) commonly found in Java, Indonesia. The specific primers were derived from the *cytochrome c oxidase subunit 1 (COI)* gene, designed using the SP-Designer V7.0 application, and validated using both in silico and *in vitro* methods. The validation results demonstrated that all five pairs of primers were highly specific, generated correct amplicons, and successfully detected the five distinct species present in a Javan mongoose feces sample. These findings are significantly important as they enable the effective detection of rat and mouse species and potentially provide valuable ecological insights from the field.

KEYWORDS Cytochrome c oxidase subunit 1 (COI); DNA; Mouse; Non-invasive; Rodents

1. Introduction

The Muridae family, comprising rats and mice, is a vital component of ecosystems, with their activities having significant impacts on biodiversity and health ecosystem (Russell and Holmes 2015), and food security (John 2014). While eradication measures may be warranted in certain situations, preserving their populations is equally important since they support the dispersion of plants (Chen et al. 2023) and fungi (Elliott et al. 2020) or serve as a source of food for meso-carnivores (Xiong et al. 2017). To effectively manage populations and protect native biodiversity, it is crucial to have a comprehensive knowledge of a species' demographics, ecology, and impact, as highlighted by Browett et al. (2020). This is particularly important in regions such as Indonesia, where rat and mouse invasions pose a significant challenge and require a deeper understanding of these animals' ecological interactions to mitigate the negative impacts. Accurate identification of these species is the key to the understanding.

Identification of rat and mouse species is challenging because of their high diversity, thus requests molecular approaches. The old-world rats and mice (subfamily *Murinae*) consist of at least 550 species worldwide (Musser and Carleton 2005), 168 species distributed in Indonesia, and 22 of which can be found in Java (Maryanto et al. 2019). Some members are closely related. Identifying them based on their morphology has become quite challenging (D'Elía et al. 2019) and is usually only done by specialized taxonomists who are rarely accessible. Moreover, the morphological method is a successful identification technique that is applicable solely to complete samples. Identifying parts of the body such as blood, tissue, feces, skin, bones, and hair can be quite challenging. An advanced method is available for identification even with a limited sample. Using DNA sequence as a marker eliminates the need for taxonomical expertise and specific sample forms (Bohmann et al. 2014).

The application of the molecular approach needs to compromise between expected data quality and available resources. The classical approaches typically depend on the sequencing technique, which requires a substantial investment of equipment, human resources, and time, although is still less resources-demanding when compared to the recent metabarcoding technique (Mori and Matsumura 2021). Considering the limitation of resources in Indonesia, a technique of species detection without sequencing

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can be adopted for rapid surveillance. The detection involves polymerase chain reaction (PCR) analysis, which uses specific primers that amplify only the DNA of the targeted species. Although it requires an initial investment in the robust design of a PCR primer for each species, the cost of the whole analysis will be minimal.

Several studies have previously reported specific PCR primers for identifying rats and mice. A set of PCR primers has been developed to quickly detect *Rattus norvegicus* and *Mus musculus* (Martín et al. 2007). Additionally, other PCR primer sets have been developed to identify *Bandicota indica, Milardia meltada, Rattus rattus* and *Tatera indica* (Lakshminarayanan et al. 2015). However, those primer sets are not practical for identifying the rat and mice species in Java due to unmatched taxa or reliance on sequencing data for species identification. This may be prohibitive for surveillance involving a large sample size or short time.

In this paper, an improved molecular approach for identifying 5 species of rats and mice commonly found in Java, Indonesia (*Bandicota indica*, *Rattus tiomanicus*, *Rattus argentivente*, *Mus musculus*, and *Rattus tanezumi*) is reported. The first three species draw attention because they were abundant and frequently reported to cause agricultural damage and health problems (Herawati and Sudarmaji 2021), meanwhile, the last two species were potential leptospirosis transmitters in Java (Marbawati et al. 2016). Therefore, reliable information about these species including their identity is urgently needed for population management purposes to mitigate the problems. Particularly, this study aimed to design and validate a PCR primer set to practically detect those rats and mice species from degraded samples. The primer sets were derived from the *cytochrome c oxidase subunit 1 (COI*) gene as its sequence exhibits distinct variations across a diverse range of animal phyla (Hebert et al. 2003) and has the same power as commonly used *cytochrome b* gene for identifying species in mice, voles, and shrews (Pfunder et al. 2004). For a more thorough validation, we trialed the primer by involving it in a PCR using DNA extracted from the feces of the Javan mongoose (*Urva javanica*) as a template following primer validation using positive control. The mongoose is a known predator of rats, mice, and other small creatures; therefore, its feces most likely contain a mix of DNA of various animals. It mimicked the field situation and therefore represented an ideal condition for testing the primer specificity.

2. Materials and Methods

2.1. Primer design and validation

PCR primers were designed carefully from published sequences of the *COI* gene, adhering to strict rules. To ensure that they were unique, three sequences of each species of the rats, mice and Javan mongoose were obtained from the GenBank of the National Center for Biotechnology Information (NCBI) database. These sequences were aligned using MEGA 7 (Tamura et al. 2011) and then inputted into SP-Designer V.7.0 (Villard and Malausa 2013) for primer design. The designed primers were intended to amplify unique and short (100–500 base pairs) DNA fragments for each species, which was suitable for degraded DNA samples. The primers' specifications included a length of 18–24 base pairs, a Guanine-Cytosine content between 40–60%, and a melting temperature within the range of 55–65 °C. It was important to avoid the formation of secondary structures such as hairpins and dimers, as they can reduce the efficiency of the PCR process by hindering the primers' extension.

The designed primers were tested via in silico and *in vitro* methods. In silico testing was performed using the Primer-Blast feature of NCBI, and the results were used as a basis for the initial screening of the designs. This was performed to prevent the use of failed subsequent designs for the *in vitro* test. These *in vitro* tests were conducted using touchdown-PCR to improve specificity. Furthermore, the PCR cycle was set up according to the protocol of a pre-mixed PCR reagent (MyTaqTM HS Red Mix 2×, Bioline Inc, London, UK), with adjustment of the annealing temperature, time, and the number of cycles as shown in Table 1. The PCR was conducted on a total volume of 40 µL, consisting of 20 µL the pre-mixed PCR reagent, 0.5 µM of forward and reverse primer, and 12–20 ng of the DNA template. Furthermore, this template was isolated from tissues of each rat and mouse species for positive control and from Javan mongoose feces as a trial.

To evaluate the results after PCR, gel imaging and sequencing analysis were employed. The amplicon was visualized using electrophoresis on a 0.5× TBE buffer and 2% agarose gel, with Ethidium Bromide dye (0.5 µg/mL), at 100 Volts for 50 min. A 100 bp marker (ExcelBandTM, SMOBiO Inc, Hsinchu, Taiwan) was used as a ladder. The electrophoresis results were observed using a Kodak Gel Logic Imaging System. Amplicons that produced clear bands were further analyzed using the Sanger sequencer (The Applied Biosystem 3500 Genetic Analyzer; Thermo Fisher, US) using one direction mode. Chromas (http://technelysium.com.au/wp/chromas) and BioEdit (Hall 1999) were used to process the sequencing results. The DNA sequences were then aligned with the homologous sequences found in the GenBank of NCBI databases to identify the results.

2.2. DNA sampling

DNA was collected from tissue samples of a single animal from each species for conducting *in vitro* testing. These animals were live trapped in Southern Yogyakarta's forested areas, particularly in Wanagama (7.89° S, 110.54° E) and Wonosadi (7.82° S, 110.68° E) forests, between March and October 2018. Additionally, feces of Javan mongoose were also collected from the same areas. The entire process was carried out following ethical guidelines. DNA was isolated from a small tip of the rat's tail using a DNAEasy blood and tissue kit (Qiagen, Hilden, Germany) as per the provided protocol, and served as a positive con-

TABLE 1 Touchdown PCR cycle profile.

Stages	Temperature (°C)		Time	Cycles
	A	B		
Stage 1:				
Initial Denaturation	95	95	2 min	1
Stage 2:				
Denaturation	95	95	15 s	
Annealing	57 (Δ°C = -0.5)	52 (Δ°C = -0.5)	15 s	10
Elongation	72	72	10 s	
Stage 3:				
Denaturation	95	95	15 s	
Annealing	52	47.5	15 s	15
Elongation	72	72	10 s	
Stage 4:				
Denaturation	95	95	15 s	
Annealing	52 (Δ°C = -0.5)	47.5 (Δ°C = -0,5)	15 s	10
Elongation	72	72	10 s	
Stage 5:				
Post Elongation	72	72	1 min	1
Hold	4	4		1

A = *Mus musculus* and *Rattus tanezumi* primers; B = *Rattus tiomanicus*, *Rattus argentiventer*, and *Bandicota indica* primers.

trol for PCR.

Meanwhile, DNA from Javan mongoose feces was isolated using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), preceded by a pre-treatment of incubating 0.8–1.6 grams of feces in a 5 mL ASL buffer (Qiagen, Hilden, Germany) at 65 °C for one hour to promote cell lysis. After processing the samples following the manufacturer's protocol, DNA was extracted and quantified using a Qubit 2.0 fluorometer. Samples with a concentration of less than 1 ng/µL were excluded. The extracted DNA was then further processed for animal metabarcoding, using the methodology reported by Subrata et al. (2021), and served as a template for PCR trials.

3. Results and Discussion

In this study, the primers used successfully generated a short amplicon of 141–370 bp in length (Table 2). The designed primer set consistently performs well in both in silico and *in vitro* tests. According to the tests, the primers resulted in sufficient productivity and specificity of the amplicons. Productivity was verified by an *in vitro* test, which showed that the PCR produced amplicon in sufficient quantity and appropriate length. The appearance of clear DNA bands in the gel imaging provides evidence of sufficiency, although the quantity varied as shown by the thickness of the DNA band in Figure 1.

Furthermore, the gel visualization indicates that the DNA of the target species was successfully amplified, as demonstrated by the visible bands (refer to Figure 1a, b, and c). These bands were observed in multiple Java mongoose feces samples, and their ladder length was similar to that of the specific primer pairs (as detailed in Table 2). Specifically, the MMU primers produced amplicon bands of approximately 200 bp, while the RTA primers generated bands of about 150 bp. The RTI primers resulted in bands of 350–400 bp, the BIN primers produced bands of 250 bp, and the RAR primers yielded bands of approximately 150 bp. These band lengths were consistent with those obtained from the positive control.

The primers showed good specificity, as evidenced by the results of in silico and *in vitro* tests. The initial in silico test showed that the primers could recognize the target sequence and complement it accordingly. However, they induced the formation of secondary structures, as evidenced by the value of the column's intrinsic (hairpin) and intrinsic 3′ complementarity (primer–dimer) (Table 3). Furthermore, self-complementarity values ranged from 2.00 to 6.00, while intrinsic 3′ values (forward RTA, reverse RTA and RTI) were around 2.

The specificity of the primers used for testing was later confirmed through *in vitro* tests. An amplicon sequencing process was conducted to validate the specificity of the primers, and the results were compared to the GenBank of the NCBI database. The samples selected for testing (MMU-S12, RTA-S25, RTI-G8, BIN-S40, and RAR-S8) were derived from DNA templates found in Javan mongoose feces. The analysis confirmed that these samples are identical to the organisms stored in the GenBank of the NCBI database, sharing a high degree of homology (>98%). This finding is consistent with the primer specifications and in silico test results.

TABLE 2 Specific PCR primer designed for detecting 5 rat species commonly found in Java.

Primer ID	Species	Sequence	Amplicon (bp)
MMU	*Mus musculus*	Forward: 5′-AGGAGCAGGAACAGGATGAA-3′ Reverse: 5′AATAAGTACGGATCAGACAAATAGTGGAG-3′	211
RTA	*Rattus tanezumi*	Forward: 5′-TGGAGCCCCTGATATAGCATT-3′ Reverse: 5′-CCGGCTAAGGGTGGGTAT-3′	141
RTI	*Rattus tiomanicus*	Forward: 5′-GGGAACAGCCTTAAGTATTTTAATTCG-3′ Reverse: 5′-TAGGTGAAGGGAGAAAATGGT-3′	370
BIN	*Bandicota indica*	Forward: 5′-CGAGCCGAATTAGGGCAA-3′ Reverse: 5′-TGGATGATGCTAAAAGAAGAAGA-3′	238
RAR	*Rattus argentiventer*	Forward: 5′-CATCAATATAAAACCTCCTGCTATG-3′ Reverse: 5′-TAGGTTTCGGTCTGTGAGAAG-3′	142

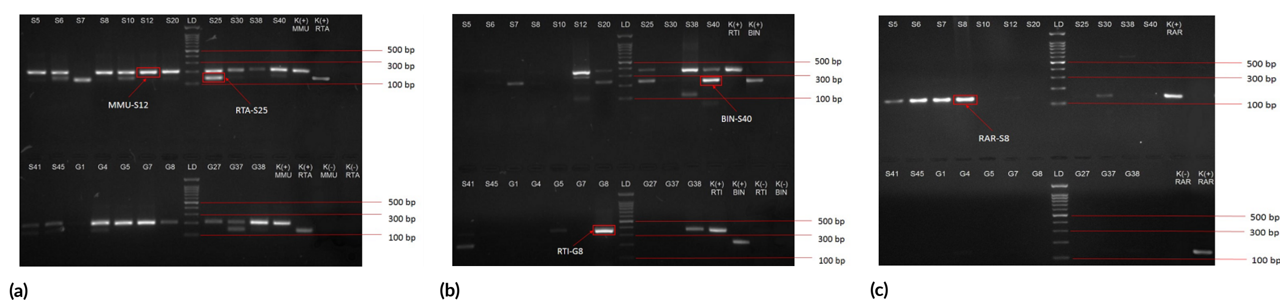

FIGURE 1 Agarose gel visualization of DNA bands of *Mus musculus* (MMU) and *Rattus tanezumi* (RTA) (a), *Rattus tiomanicus* (RTI) and *Bandicota indica* (BIN) (b), and *Rattus argentiventer* (RAR) (c). Two bands in multiple lines indicate two species are present in the samples. LD = ladder 100 bp, K (+) = positive control, K (-) = negative control, S = Javan mongoose feces samples from the Wonosadi Forest, and G = from Wanagama Forest.

TABLE 3 In silico tests of designed primers showed good specificity with the possibility of a hairpin and dimmer formation.

Primer		Species Target	Blast Result	Self-Complementarity	Self 3′ Complementarity
MMU	F	*Mus musculus*	Specific	2.00	0.00
	R			4.00	0.00
RTA	F	*Rattus tanezumi*	Specific	6.00	2.00
	R			4.00	2.00
RTI	F	*Rattus tiomanicus*	Specific	6.00	2.00
	R			2.00	0.00
BIN	F	*Bandicota indica*	Specific	6.00	0.00
	R			3.00	0.00
RAR	F	*Rattus argentiventer*	Specific	4.00	0.00
	R			3.00	0.00

Notes: F = *forward*, R = *reverse*, Specific = The blast results are consistent with the target species of the primer.

TABLE 4 Identity of amplicon sequence as checked against the GenBank of the NCBI for each rat and mouse species.

Amplicon ID	Percent of Identity	Species	Accession Number
MMU-S12	100%	*Mus musculus*	MH727419.1
RTA-S25	100%	*Rattus tanezumi*	MH303532.1
RTI-G8	100%	*Rattus tiomanicus*	KP876560.1
BIN-S40	98%	*Bandicota indica*	KT029807.1
RAR-S8	100%	*Rattus argentiventer*	FR775831.1

3.1. Discussion

A practical molecular approach was successfully developed and validated to identify five rats and mice species.

Using specific PCR primers in combination with gel imaging, the species could be quickly detected based on the distinct amplicon length. The length can be observed using a variety of methods including agarose gel, polyacrylamide, and capillary electrophoresis. The latter method leads to detailed measurements of amplicons but requires sophisticated equipment. Meanwhile, the first two methods can be performed quickly and inexpensively in a basic molecular biology laboratory. It represents a cost-effective alternative for the early detection of rats and mice, even when working with suboptimal samples.

This study is concerned with suboptimal samples because of the high possibility of encountering them when identifying rats and mice in the field. Considering the challenge of obtaining ideal genetic samples on-site, alternative materials such as blood, tissue, feces, skin, bones, and hairs can be used. While suboptimal, these materials can still be utilized for species identification through a well-designed PCR primer set. The key is to select a primer capable of producing amplicons with sufficient quantity and specificity from those suboptimal samples, which are often of poor quality, contaminated, and contain fragmented and mixed DNA (Linacre and Tobe 2013). To address this issue, primers that can amplify short amplicons without sacrificing specificity are preferred. Compared to previous rat-specific PCR primers (Martín et al. 2007; Lakshminarayanan et al. 2015), the advantage of the primers used in this study is that they produce shorter amplicons, increasing the likelihood of successfully producing target DNA from suboptimal samples, thus productive. This finding highlights the effectiveness of primers that generate relatively short amplicons.

Furthermore, the finding also reflects the ability of specific primers to amplify both good DNA templates from rat and mice tissues (positive control) and poor DNA templates isolated from the feces of Javan mongoose (trials). This is consistent with primer specifications and in silico test results. In addition, this finding also confirmed the work of Subrata et al. (2021) examining prey identification in the feces of the Java mongoose using high-throughput sequencing. Unlike DNA from the tissue, the DNA isolated from feces is a complex mixture of multiple organisms, both predatory and prey (Taberlet et al. 2018), therefore amplifying specific markers from this kind of DNA is challenging in terms of productivity and specificity. This specificity is important because it affects the accuracy of the primer in recognizing and complementing the target sequence. It also has a strong impact on the accuracy of the amplicon generated by the PCR process, as non-specific amplicons are considerably avoided by the PCR process. This result provides convincing evidence of the reliability of this approach.

Overall, the tests showed that the primers had sufficient productivity and specificity as they were able to amplify the DNA template and generate specific amplicons. This specificity is particularly important when working with mixed DNA samples such as feces or other environmental samples. As in our case, the primer sets are robust and capable of recognizing rats and mice, facilitating further collection of environmental data. In addition, the test also demonstrates the feasibility of this approach for the economical detection of rats and mice in environmental samples. Even the cost can be minimized by using primer sets in multiplex PCR. This advantage facilitates surveillance for early detection of rats and mice, and population management purposes, both eradication and biodiversity conservation.

When performing multiplex PCR, it's crucial to be cautious, especially when selecting primers. Table 2 shows that the RTA and RAR primers produce amplicons of similar lengths (141 and 142 base pairs, respectively). Similarly, the MMU and BIN primers have comparable results, generating 211 and 238 base pairs, respectively. These small differences in amplicon length can't be detected through agarose gel imaging. Therefore, it's recommended to avoid pairing RTA-RAR or MMU-BIN primers in multiplex PCR. However, the MMU and RTA combination is suitable for multiplex PCR since both primers have identical temperature profiles (as per Table 1), and their amplicons are distinguishable (as observed in Figure 1 samples S6, S10, S20, S25, S40, S45, G64, and G37). The same applies to BIN, RTI, and RAR primers.

While this primer set is productive and specific, it does have the drawback of potentially forming secondary structures, which can impede the generation of target amplicons during PCR. Fortunately, there are methods to mitigate this issue, such as implementing touchdown PCR (Moezi et al. 2019). In this present study, we employed the two-phase touchdown PCR technique (Korbie and Mattick 2008). By reducing the annealing temperature range by 0.50C across a 100C span, this method proves especially valuable when multiple primers with varying annealing temperatures are utilized in the PCR process. For more sensitive results, it is recommended that this primer set is further adjusted and validated, particularly for quantitative PCR applications.

4. Conclusions

These PCR primers are designed to provide practical and accurate detection of five rats and mice species commonly found in Java. A final test showed that these primers could recognize the species in Javan mongoose feces. The PCR primers also showed good productivity, as shown by clear DNA bands in the gel image of the corresponding length. The primers allow quick and efficient identification of the species with basic molecular equipment and only a tiny sample. It supports identifying multiple species of the species and facilitates the collection of their ecological data.

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Authors' contributions

PY and SAS designed and provided resources for the study. SRTS conducted field and laboratory work. PY, SRTS and SAS analyzed the data and wrote the manuscript.

Competing interests

The authors stated that they had no competing interests while conducting this study.

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