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RESEARCH ARTICLE

Analysis of Morphological and Genetic Diversity of Fruit Flies (*Bactrocera* spp.) on Chili Plants in East Java, Indonesia

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Abstract: Fruit flies of the genus *Bactrocera spp.* are important pest that infests many horticultural crops, including chili. With a significant economic impact, the genetic diversity and population dynamics of this pest have not been widely explored. The purpose of this study was to understand the diversity of fruit flies that infest chili in East Java, Indonesia. Morphological characterization was validated by genetic analysis of mt-COI DNA sequences. Infested chili fruits were collected from 18 locations in 6 production center districts. The results of morphological identification obtained the species *Bactrocera dorsalis* and *Bactrocera carambolae*. Genetic validation of representative DNA sequences showed high homology with mt-COI sequences from two species, namely: *B. dorsalis*, with 99.41% similarity to accession MG689254.1, and *B. carambola*e, with 99.85% similarity to accession KF659631. These results can confirm the identification of fruit fly samples morphologically. However, inter- and intraspecies genetic variations between *B. dorsalis* and *B. carambolae* show high diversity, which allows for similarities in both species. Groups *B. dorsalis* and *B. carambolae*. The study samples have their own groups that are different from the same species from other sequences that have been reported. This shows variation due to geographical factors. Morphology-based identification is an effective approach for fruit flies. However, integrated barcoding, a combination of morphological taxonomy and molecular methods, improves the accuracy and validation of identification results.

Keywords: Chilli, *Carambola, Dorsalis*, DNA Barcoding, Genetic Diversity

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Introduction

Fruit flies (Diptera: Tephritidae) are one of the major pests that threaten horticultural production globally, with economic losses reaching billions of dollars each year [1]. In Asia, the genus Bactrocera has become a serious threat to fruit and vegetable commodities including chilies (Capsicum sp.), with damage levels reaching 30-80% depending on the variety, season, and location [2, 3].

Indonesia, as a tropical country, faces serious challenges from fruit fly attacks, especially in horticultural production centers such as East Java. The complexity of this problem is further increased by the diversity of species. Bactrocera is highly adaptable to various hosts [4, 5]. Accurate species identification is key to developing effective control strategies, but this is often hampered by limitations in conventional morphological identification.

Morphology-based taxonomy, which has been the standard for decades, faces several challenges, including the availability of taxonomists, variation in insect metamorphosis stages, and the existence of complex species that are difficult to distinguish morphologically [6]. DNA barcoding, particularly the use of the Cytochrome Oxidase I (COI) gene, has been introduced as an accurate and rapid species identification approach. This method is capable of providing high resolution in distinguishing sequence variations at the inter- and intraspecific levels [7, 8].

Although several studies have been conducted on fruit flies in Indonesia, comprehensive research integrating morphological and molecular analysis on fruit fly populations infesting chili is still limited. Previous studies have focused more on morphological identification [9] or separate molecular analysis [10], while an integrated approach is still rarely used.

This study aims to analyze the diversity of fruit fly species that infest chili plants in East Java. Identification of the first generation of fruit flies with an integrated approach of morphological characterization validated using genetic analysis based on mt-COI DNA sequences. The results of this study are expected to provide a more comprehensive understanding for the development of sustainable fruit fly control strategies.

Materials and Methods

Study Location and Sample Collection

Surveys and sample collection were conducted at 18 locations from 6 chili production center districts in East Java (Batu, Blitar, Jombang, Kediri, Malang, and Nganjuk) from January to June 2024. Sampling was carried out using the method of purposive sampling based on the level of fruit fly infestation. At each location, chili fruits showing symptoms of fruit fly infestation were collected and maintained in the laboratory. Sampling locations were selected with a minimum distance between locations of 2 km to avoid sampling from the same population.

Maintenance Procedures in the Laboratory

Chili fruits were maintained in plastic containers with gauze ventilation (15x0 cm), which were given a sterile sand base with a humidity of 5-8% as a pupal medium. Maintenance was carried out under standard laboratory conditions (25±2°C, RH 60-70 %) following the protocol of [11]. Observations were carried out every day to record the time of pupae and imago emergence. Metamorphosis of fruit flies takes 12-18 days from larvae to pupae. The formed pupae are transferred to a 10 cm diameter petri dish and placed in a 30x30x40 cm cage. The emerging imago are given sterile water to drink (soaked in foam) and food (sugar in a cup) for 4-7 days after reaching full morphological development; species identification is carried out [12].

Morphological Characterization

Morphological identification was performed on first-generation fruit fly specimens using a stereomicroscope (Nikon SMZ1000). Characterization refers to the standard identification key for Bactrocera spp. The diagnostic characters observed include the thorax, wings, and abdomen. Species *B. dorsalis* has a red or reddish-brown thorax; the abdomen has a clear T pattern; the anterolateral corner of the terga is triangular; the wings have a costa band line following R2+3 extending to the tip. While B. *carambolae* has: The thorax is opaque black with yellow bands, the abdomen has a T pattern with narrow bands, the anterolateral corner of the terga is square, the costal band of the wings exceeds R2+3 extending to the tip [11].

DNA extraction, PCR Amplification, and Sequencing

The haplotype individuals of fruit flies from morphological characterization were verified through the total DNA extraction process. Extraction was carried out using the gSYNCTM DNA Extraction kit (Geneaid, GS300) following the manufacturer's protocol. Total DNA extraction results were measured for quantity and quality on a NanoDrop spectrophotometer. The next stage is PCR amplification of the COI gene target using MyTaq HS Red Mix, 2x kit (Bioline, BIO-25048) with universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') at a target gene size of 700 bp [13]. PCR amplification settings include initial denaturation at a temperature of 95oC for 1 minute,

annealing at 52oC for 15 seconds, and final extension at 72oC for 15 seconds. The cycle lasted 35 times. The amplification product was electrophoresed on a 1% agarose gel in TBE buffer for 30 minutes at 100 volts and visualized using a UV illuminator. The results were continued with two-way DNA sequencing using the Sanger DNA Sequencing (Capillary Electrophoresis) method carried out in the testing Lab.

Quality Control and Validation

For genetic verification, bioinformatics analysis was performed. The results of forward and reverse sequencing were verified and annotated for alignment and phylogenetic tree construction. Annotated and ambiguous sequences were removed from the primary sequence to obtain a consensus sequence [14]. The annotation process was performed using BioEdit software [15]. The results of the consensus sequence were then subjected to BLAST analysis to view homology in the GenBank database and phylogenetic analysis. 10 accession numbers were selected and downloaded for each target species, *B. dorsalis* and B. *carambolae*, based on the COI gene and aligned using the ClustalW algorithm [16]. The aligned DNA distance matrix was obtained using the Tamura-Nei model [17], and the phylogenetic tree was constructed using the Neighbor-Joining (NJ) algorithm [18]. All steps of the phylogenetic analysis were performed using MEGA X with 500-fold bootstrapping support [19].

To ensure the accuracy of the results, morphological identification was validated by a fruit fly taxonomic expert (Dr. Suputa, Basic Entomological Laboratory, Faculty of Agriculture, Universitas Gadjah Mada, Indonesia); positive and negative controls were used in each PCR analysis, and sequencing was performed in both directions for verification. Specimens were stored in the laboratory for future reference.

Data Analysis

Morphometric data were analyzed using SPSS version 25.0 to calculate morphological variation between populations, and the relationship between genetic and geographic distances was analyzed using the Mantel test with 10,000 permutations.

Results

Taxonomy of Chili Fruit Flies Based on Morphology

The results of the study showed the presence of two species of fruit flies that infest chili plants in East Java, namely Bactrocera *dorsalis* and Bactrocera *carambolae*. *B. dorsalis* was found as the dominant species because it was found in 18 research locations and infested all types of chili peppers studied (large chili peppers, curly chili peppers, and cayenne peppers). On the other hand, B. *carambolae* has a more limited distribution, found only at 8 research locations (Table 1). Dominance of infestation by *B. dorsalis* is shown not only in its distribution in all research locations, but also by the higher proportion of *B. dorsalis*. From a total of 398 individual fruit flies collected, *B. dorsalis* dominated as many as 348 individuals (87.4%), while B. *carambolae* was represented by only 50 individuals (12.6%).

Table 1. Fruit fly species found on chili fruit in East Java

Code Location	Plant host	Fruit Fly Species		Amount Fruit Fly
		B. dorsalis	*B. carambolae*	
L01	Cabai Besar	43	-	43
L02	Cabai Keriting	34	8	42
L03	Cabai Rawit	20	-	20
L04	Cabai Besar	28	8	36
L05	Cabai Keriting	7	3	10
L06	Cabai Rawit	9	-	9
L07	Cabai Besar	19	4	23
L08	Cabai Keriting	14	-	14
L09	Cabai Rawit	8	5	13
L10	Cabai Besar	25	6	31

L11	Cabai Keriting	12	-		12
L12	Cabai Rawit	7	4		11
L13	Cabai Besar	17			17
L14	Cabai Keriting	8	-		8
L15	Cabai Rawit	10	-		10
L16	Cabai Besar	51	12		63
L17	Cabai Keriting	18	-		18
L18	Cabai Rawit	18	-		18
	N	348 (87,4%)	50	(12,6%)	398 (100%)

Description: L01-L03 (Batu), L04-06 (Blitar), L07-L09 (Jombang), L10-L12 (Kediri), L13-L15 (Malang), and L16-L18 (Nganjuk)

Validation of Morphological Identification by Analysis of mt-COI Sequence Diversity

BLAST analysis of COI gene sequences of randomly selected individual fruit flies from representative sample collection locations showed high homology with mt-COI sequences from two species, namely: *B. dorsalis* (Percentage Identity 99.41% and 99.56% with accession MG689254.1) (Figure 1) and B. *Carambolae* (Percentage Identity 99.85 and 100% with accession KF659631.1) (Figure 2). These results can confirm the identification of fruit fly samples morphologically. However, inter- and intraspecies genetic variations between *B. dorsalis* and B. *carambolae* show high diversity. This is indicated by the E-value (Expectation value), whose values are close to zero in the top 10 BLAST hit accessions both in B *dorsalis* and B. *carambolae*. An E-value approaching zero indicates that there is no possibility that the alignment event occurred by chance (Table 2), so an approach with phylogenetic construction is needed to see how far the kinship distance is.

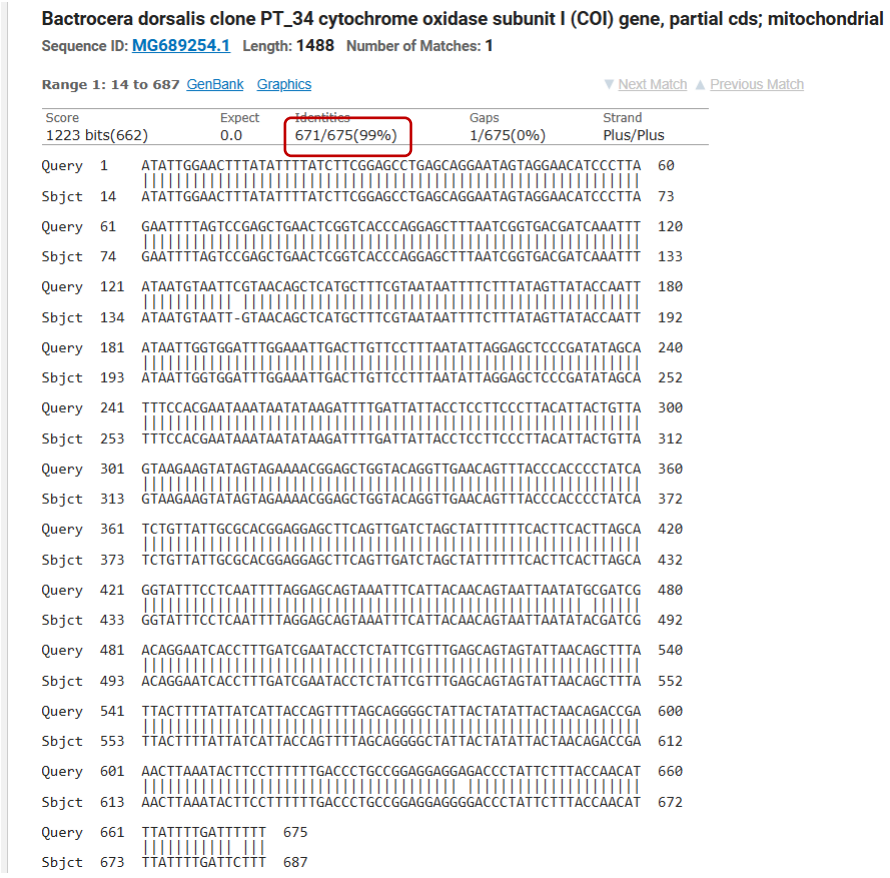

Fig. 1: Pairwise alignment of the COI gene sequence of *B. dorsalis* clone PT_34 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial (MG689254.1). Query is the study sequence (L01 & L10), and Subject is the GenBank sequence

Fig. 2: Pairwise alignment of COI gene sequence of Bactrocera *carambolae* isolate 15 Cytochrome Oxidase Subunit I (COI) gene, partial cds; mitochondrial (KF659631.1). Query is the study sequence (L04 & L16), and Subject is the GenBank sequence

Table 2: Results of sequence blasting with several different haplotype sequences of *B. dorsalis* and B. *carambolae* from GenBank

Deskripsi	Query cover	E value	Identity (%)
MG689254.1 *Bactrocera dorsalis* clone PT_34 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100%	0.0	99.41%
MG689129.1 *Bactrocera dorsalis* clone LB_37 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100%	0.0	99.26%
MG689158.1 *Bactrocera dorsalis* clone VT_19 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100%	0.0	99.26%
MG689177.1 *Bactrocera dorsalis* clone VT_38 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100%	0.0	99.26%
MG687972.1 *Bactrocera dorsalis* clone BS_50 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100%	0.0	99.26%
MT121278.1 *Bactrocera carambolae* isolate Indonesia 02 mitochondrion, complete genome	100%	0.0	99.26%
PP485741.1 *Bactrocera dorsalis* isolate PG17 mitochondrion, complete genome	100%	0.0	99.11%
KF659631.1 *Bactrocera carambolae* isolate 15 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100%	0.0	100%
MN104217.1 *Bactrocera carambolae* isolate S2 mitochondrion, complete genome	100%	0.0	99.11%

Construction of phylogenetic trees using the Neighbor-Joining (NJ) method with repetition (bootstrapping) 500 times was performed on each sample group. In L01 and L10, it was shown that the sequences formed one group with a very close distance (0.0000) with a bootstrap value > 50% and were closely related, having the same ancestor node as the sequences. *B. dorsalis* was previously recorded with accession numbers MH187231 and MH187230 with bootstrap values > 50% (Figure 3). This illustrates the group *B. dorsalis*. The study sample has its own uniqueness or group. Meanwhile, in samples L04 and L16, one group was also formed with a bootstrap value > 50% and was related to the same node as the sequence group. *B. carambolae* (KF659630, KF659628, and KF659636) even though the bootstrap value was < 50% (Figure 4).

Molecular validation through mt-COI gene analysis strongly supports the morphological identification results. Genetic distance and clustering patterns provide clear evidence of the existence of two species. Bactrocera species that attack chili peppers in East Java, with each species showing consistent morphological and molecular characteristics across sampling locations.

This integrated approach combining detailed morphological characterization and molecular analysis provides strong evidence for species identification and reveals patterns of genetic diversity in fruit fly populations affecting chili production in East Java.

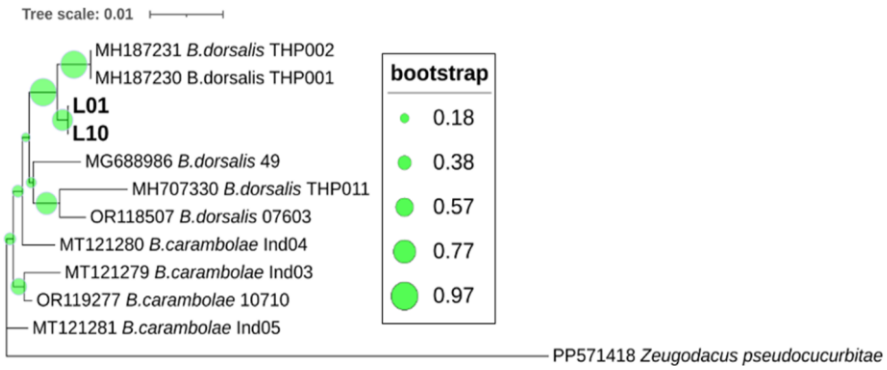

Fig. 3: Phylogenetic tree of fruit fly relationships based on mitochondrial cytochrome oxidase I (mt-COI) genes. L01&L10 are the sequences studied

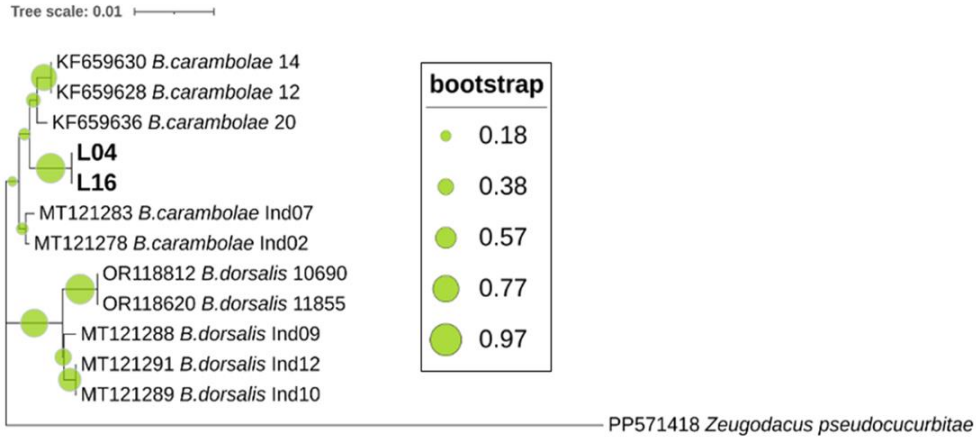

Fig. 4: Phylogenetic tree of fruit fly relationships based on mitochondrial cytochrome oxidase I (mt-COI) genes. L04&L16 are the sequences studied

Discussion

Validation of Morphological and Molecular Species Identification. A comprehensive survey of chili production centers in East Java revealed the presence of two species, Bactrocera dominant: *B. dorsalis* and B. *carambolae*. Morphological identification of first-generation specimens showed consistency of diagnostic characters that distinguish these two species. This approach, which integrates morphological and molecular data, strengthens the accuracy of species identification, in line with previous studies that emphasize the importance of integrated taxonomic validation.

Molecular techniques for taxonomic identification are often referred to as the DNA barcode [20]. In a series of stages of recognizing nucleotide sequences, one of the functions of BLAST is to identify genes in new (unknown) genomes through sequence alignment by comparing them with known genes in the database [21, 22]. The sequences L01, L10, L04, and L16 from the PCR amplification process against the representative mt-COI target gene have high homology with the mt-COI sequence *B. dorsalis* (99%) with MG689254.1 and B. *carambolae* (100%) with KF659631.1. In many disciplines, obtaining accurate taxonomic identification is a must. GenBank is the primary public repository of sequences. Barcode DNA. Results of the multilocus barcode approach increase the success of identification [23]. An identity percentage equal to or greater than 97% indicates a high probability of identifying specimens in the same species. Likewise, [24] emphasized that for microbial identification, an identity percentage above 97.5% supports the determination of the species level.

Although the query sequence has high homology to the GenBank database sequences, the e-value (Expectation value) significantly approaching zero occurred in both species *B. dorsalis* and *B. Carambolae* (Table 2). This gives the impression that the query sequences are ambiguous in other species. According to [25], a low e-value indicates a strong match, but the significance threshold can vary according to the context of the researcher's goal or study. In addition to considering the e-value, this study aims to measure the distance matrix, which is manifested in the phylogenetic tree and bootstrapping. Although this study only analyzed partial sequences, namely the mtCOI gene, [26] conducted a comparative study of complete mitochondrial sequences of B. *carambolae*, as B. *carambolae*is one of the sibling species of the complex *B. dorsalis* and is considered to be very closely related to *B. dorsalis*, where the analysis results revealed several species-specific polymorphic sites. One of the polymorphisms in the COI gene is located at positions 425 and 933, respectively, in the form of nucleotides "C" and "G" in *B. dorsalis*, while the nucleotides “T” and “A” in B. *carambolae*. Genetic confirmation was also studied by [27] on several Bactrocera genera. B. *carambolae* emerged as a distinct monophyletic clade, whereas *B. dorsalis, B. papayae*, and B. philippinensis did not. It is not yet clear who the ancestors are or whether they are the same biological species [27].

The construction of the phylogenetic tree was carried out in two parts based on each

group of query sequences against 10 selected database sequences and 1 outgroup sequence (species Zeugodacus pseudocucurbitae). This is related to better understanding the taxonomy and evolution of Dacini to build a robust phylogeny and test the phylogenetic relationships between the genus, subgenera, and species complexes [28]. The first phylogenetic tree is for L01 and L10, while the second is for L04 and L16. In both phylogenetic trees, the query sequences form one group with a very close distance (0.0000) and a bootstrap value > 50%. This indicates that the level of intraspecific diversity in the query is relatively low. This value indicates a stable population with high gene flow between sampling locations [29]. This is similar to the findings in the population of Bactrocera in other Asian regions.

Geographic Distribution Pattern

The distribution patterns of both species show variation based on altitude, with *B. dorsalis* more dominant in the highlands and B. *carambolae* in the lowlands. This pattern indicates different habitat preferences, possibly related to specific ecological adaptations. Low genetic diversity within the population but widespread indicates high dispersal ability, both naturally and through the movement of infested fruits [1]. Regarding distribution patterns, [30] revealed the population structure and global invasion routes of *B. dorsalis* across Asia, Africa, and Oceania, and identified loci in the genome responsible for thermal adaptation. That *B. dorsalis* originated from the South Indian region with three independent routes of invasion and spread throughout the world:

(1) From North India to North Southeast Asia, then to South Southeast Asia

(2) From North India to North Southeast Asia, then to China and Hawaii

(3) From South India to mainland Africa, then to Madagascar, which was largely facilitated by human activities including trade and immigration. Cyp6a9 gene expression facilitates thermal adaptation in *B. dorsalis* and increases its invasion

Implications for Integrated Pest Management

Confirmation of the existence of two species of Bactrocera with different habitat characteristics and preferences has important implications for control strategies. Integrated control strategies need to consider local species composition and distribution patterns to optimize effectiveness. Low genetic diversity within populations suggests that control strategies between sites may be effective, but coordination between regions is still needed given the high gene flow.

Conclusion and Suggestions

Morphological-based diversity identification studies validated with mt-COI gene barcodes indicate the presence of two species of Bactrocera (*B. dorsalis* and B. carambilae) on chili plants in East Java. Validation of morphological data with mtCOI gene Analysis showed that barcode technology and morphology-based identification are effective approach for fruit flies. However, integrated barcode, a combination of morphological taxonomy and molecular methods, improves the accuracy and validation of the results. Low but widespread intraspecific genetic diversity indicates a stable population with high gene flow between locations. Overall, this study provides important information about the diversity of fruit fly Species on chili plants in East Java.

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Author's Contributions

Rudi Cahyo Wicaksono: Contributed to conceptualization, data curation, investigation, formal analysis, and manuscript drafting.

Bambang Tri Raharjo, Akhmad Rizali, Aminudin Afandi and Mizu Istianto: Contributed to supervision, methodology development, manuscript review, and editing.

Wasis Senoaji: Contributed to methodology, formal analysis, validation, and investigation. All authors read and approved the final version of the manuscript.

Ethics

The authors declare that this study did not involve human participants, vertebrate animals, or endangered species. All sampling procedures were conducted in accordance with institutional and national research guidelines. The authors are committed to maintaining scientific integrity and will promptly inform the journal editor if any significant errors or ethical issues are identified after publication that may affect the validity of the research findings.

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