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Phylogeny of two accacoeliid species (Digenea: Hemiuroidea) ex *Mola mola* (Linnaeus, 1758) (Tetraodontiformes: Molidae) from Northwest Pacific, with first molecular data on *Odhnerium* Yamaguti, 1934

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Abstract

Accacoeliid digeneans associated with fish of the family Molidae exhibit enigmatically high taxonomic diversity. However, the phylogenetic relationships between species within this digenean taxon are poorly understood. In the present study, the first nuclear *28S rRNA* gene, ITS2 region of nuclear DNA, and mitochondrial *cox1* gene sequence datasets were obtained for two members of the Accacoeliidae, a type and only species of the genus *Odhnerium* Yamaguti 1934 and an unidentified *Accacladocoelium* sp. collected from *Mola mola* (Linnaeus, 1758) off Iturup Island. Analyses of molecular differentiation and phylogenetic relationships indicate that *Accacladocoelium* sp. is a sister species to *Accacladocoelium nigroflavum* (Rudolphi, 1819). The genus *Odhnerium* is closely related to *Tetrochetus* Looss, 1912, on the *28S rRNA* gene-based phylogenetic tree. Results of phylogenetic analysis based on both the mitochondrial *cox1* gene and the concatenated ribosomal ITS2 region and *cox1* gene of mtDNA show that the genus *Odhnerium* is close to the *A. nigroflavum* + *Accacladocoelium* sp. clade. In turn, the genus *Accacladocoelium* does not have monophyletic status in the trees reconstructed from these data.

Introduction

The family Accacoeliidae Odhner, 1911 is a relatively small group of hemiuroidean digeneans (Gibson 2002). A significant portion of species in this family (10 out of 25) are host specific parasites of teleosts of the family Molidae (Tetraodontiformes), with eight of them being specific to only *Mola* spp. (Bray and Gibson 1977; WoRMS 2025). Until recently, two subfamilies were recognized within the Accacoeliidae: the nominotypical subfamily and Paracaccladiinae (e.g., Gibson 2002). However, molecular data on the genus *Paraccacladium* Bray & Gibson, 1977 suggest that the Paraccacladiinae should be considered to be promoted to the family level (Sokolov *et al.* 2021).

The genus *Mola Linck, 1790* contains only three species, the most common Ocean Sunfish *Mola mola* (Linnaeus, 1758), Bumphead Sunfish *Mola alexandrini* (Ranzani, 1839), and recently described hoodwinker ocean sunfish *Mola tecta* Nyegaard, Sawai, Gemmell, Gillum, Loneragan, Yamanoue & Stewart, 2017 (Caldera *et al.* 2020; Nyegaard *et al.* 2017). All three species are quite ecologically uniform with a very wide distribution in the ocean except polar regions. However, hoodwinker ocean sunfish is mostly recorded in the 50th latitudes of the Southern hemisphere (Nyegaard *et al.* 2017). Similarly to their host fish, a wide distribution has also been noted for many accacoeliids, particularly *Odhnerium calyptrocotyle* (Monticelli, 1893) (Bray and Gibson 1977; Manter 1954; Yamaguti 1934). However, the conspecificity of accacoeliid digenean isolates from different geographically distant parts of their range has not been tested using DNA sequence data. The significant taxonomic richness of *Mola*-associated accacoeliids constitutes one of the major evolutionary puzzles of this parasite group. Resolving this puzzle is impossible without incorporating data on the phylogeny, ecology, and biogeography of both the accacoeliid digeneans and all their hosts. Meanwhile, the phylogenetic relationships among different species and genera of the Accacoeliidae remain one of the least studied aspects. It is enough to note that molecular data are currently available for only four species of this family (Ahuir-Baraja *et al.* 2015a, 2015b; Louvard *et al.* 2024; Littlewood and Olson 2001; Olson *et al.* 2003). In this study, we focus on reconstructing the phylogeny of two accacoeliid isolates from ocean sunfish caught in the Northwest Pacific, *Accacladocoelium* sp. and *O. calyptrocotyle.*

Material and methods

One specimen of ocean sunfish was caught off Iturup Island (Kuril Islands, Russia) on 10 September 2021. The specimen (TL 60 cm, weigh 22 kg) was collected from the fixed seine installed by RUSKOR Ltd. for Pacific salmon fishing in the Odessky Bay ('Odesskiy Zaliv') at the coordinates 44°48′24″N&147°13′37″E. In the laboratory, the specimen was examined for parasites using standard techniques of parasitological dissection (Bykhovskaya-Pavlovskaya 1985; Klimpel *et al.* 2019). We found the body fragment of *Accacladocoelium* sp. (anterior extremity from the oral to the ventral suckers) and seven whole specimens of O. calyptrocotyle (Figure 1) in the intestine. The digenean worms were washed in fresh water and subsequently fixed in 70% ethanol. A small fragment (the ventral *sucker)* of *Accacladocoelium* sp. and one whole specimen of *O. calyptrocotyle* were examined *using* molecular techniques. Remaining *Accacladocoelium* sp. fragment and other *O. calyptrocotyle specimens* were stained with acetocarmine, dehydrated in a graded ethanol series, cleared with dimethyl phthalate, and finally mounted in Canada balsam. The drawings were made with the help of the camera lucida. Parasite species were identified according to Linton (1898), Timon-David and Musso (1971), and Bray and Gibson (1977). Voucher of *O. calyptrocotyle* was deposited in the Museum of Helminthological Collections of the Centre of Parasitology of the A.N. Severtsov Institute of Ecology and Evolution, RAS (IEE RAS; Moscow, Russia) numbers # 14363.

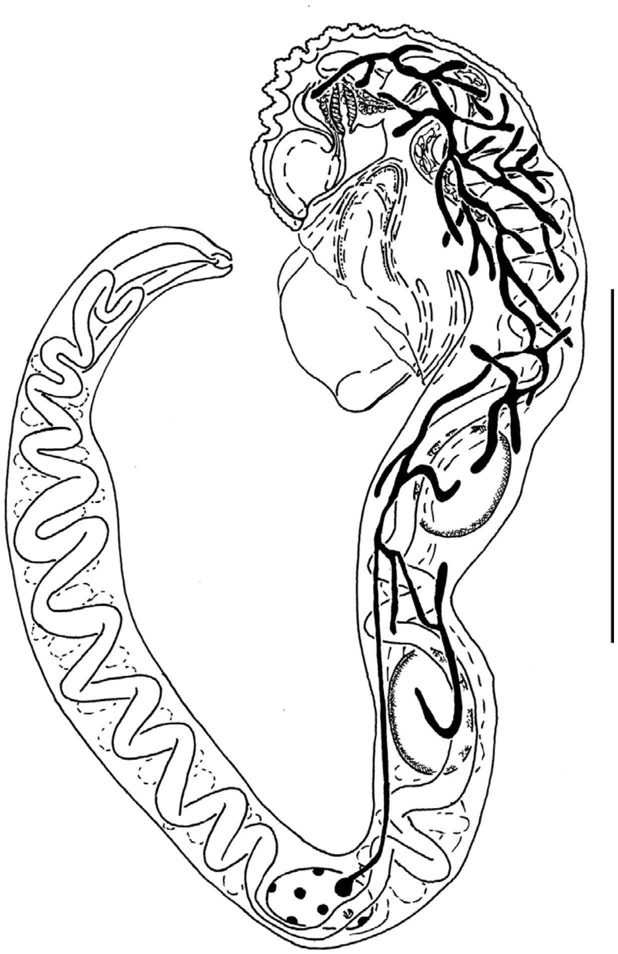

Figure 1. Paragenophore of *Odhnerium calyptrocotyle* ex *Mola mola* off Iturup Island, lateral view. Scale bar 3 mm.

DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, Toronto, ON, Canada) as per the manufacturer's instructions. The polymerase chain reaction (PCR) amplification volume amounted of 10 µl containing 5 µl GoTaq® Green Master Mix, 1 µl each primer, 1 µl DNA template, and 3 µl sterile deionised water. Fragment of the gene encoding 28S ribosomal DNA (*28S rRNA* gene) was amplified with the primers 28SA (5′-TCG ATT CGA GCG TGA WTA CCC GC-3′) (Matejusova and Cunningham 2004) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach *et al.* 2003) with an annealing temperature of 55°C. A ribosomal ITS2 fragment was amplified with primers BD1 (5'-GTC GTA ACA AGG TTT CCG TA-3') and BD2 (5'-TAT GCT TAA ATT CAG CGG GT-3') (Luton *et al.* 1992) with an annealing temperature of 54°C. Mitochondrial *cox1* gene partial sequences were amplified with PCR primers JB3 (5'-TTT TTT GGG CAT CCT GAG GTT TAT-3') and COI-R1_Trema (5'-CAA CAA ATC ATG ATG CAA AAG G-3') (Bowles *et al.* 1995; Miura *et al.* 2005). Negative and positive controls using both primers were included. Polymerase chain reaction was performed in a GeneAmp 9700, Applied Biosystems, USA.

PCR products were directly sequenced using the ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA), as recommended by the manufacturer, with the internal sequencing primers described by Tkach *et al.* (2003) for *28S rRNA* gene, and Luton *et al.* (1992) for the ITS2 rDNA fragment. The *cox1* gene fragments were sequenced with PCR primers, described above. PCR products were analysed using a GA3500 genetic analyser (Life Technologies, USA) at the FSC of Biodiversity FEB RAS. Sequences were submitted to GenBank of the NCBI database with the accession numbers described in Table 1.

The phylogenetic relationships among species of the Accacoeliidae were inferred from our data and the sequences of the other Hemiurata species obtained from the NCBI GenBank database (Table 1). Ribosomal DNA sequences were assembled with SeqScape v. 2.6 software. Alignments and estimation of the number of variable sites and sequence differences were performed using MEGA 7.0 (Kumar *et al.* 2016). Phylogenetic analyses of the nucleotide sequences were performed using the Bayesian inference algorithm with MrBayes v. 3.2.6 software (Huelsenbeck *et al.* 2001). The best nucleotide substitution models, TIM3+G and TPM1uf+G for ribosomal *28S rRNA* and mitochondrial *cox1* gene sequence fragments, respectively, were estimated with jModeltest v. 2.1.5 software (Darriba *et al.* 2012). The HKY+G nucleotide substitution model was estimated as optimal for concatenated ITS2 rDNA and *cox1* gene of mtDNA sequence dataset. Bayesian analysis was performed using 10,000,000 generations, with two independent runs. Summary parameters and the phylogenetic tree were calculated with a burnin of 2,500,000 generations. The significance of the phylogenetic relationships was estimated using posterior probabilities (Huelsenbeck *et al.* 2001).

Results

Three phylogenetic trees for the Accacoeliidae were reconstructed with Bayesian analysis based on *28S rRNA* gene fragments (834 bp), mitochondrial *cox1* gene fragments (391 bp), and concatenated ribosomal ITS2 and mitochondrial *cox1* gene fragments (704 bp), respectively. The *28S rRNA* gene-based phylogenetic tree (Figure 2) showed strongly supported monophyly for the family Accacoeliidae. The

Table 1. List of nucleotide sequences of hemiuroidean trematodes incorporated into molecular analysis. New material marked with bold

			GenBank accession number		
Species	Host	Source	*28S rRNA* gene	ITS2 region	*cox1* gene
Accacoeliidae					
Accacoelium contorum	*Mola mola*	Olson *et al.* (2003); Ahuir-Baraja *et al.* (2015b)	AY222190, KT005303	KT005304	–
	Janthina janthina	Louvard *et al.* (2024)	OR814153	–	–
Accacladocoelium macrocotyle	*Mola mola*	Ahuir-Baraja *et al.* (2015a)	–	KF687302, KF687303	KF687298, KF687300
***Accacladocoelium* sp.**	***Mola mola***	**This study**	**PV486792**	**PV486800**	**PV483242**
Accacladocoeliumnigroflavum	*Mola mola*	Ahuir-Baraja *et al.* (2015a)	–	KF687304–KF687306	KF687297, KF687299, KF687301
Odhnerium *calyptrocotyle*	***Mola mola***	**This study**	**PV486793**	**PV486801**	**PV483242**
Tetrochetus coryphaenae	*Glaucus atlanticus*	Louvard *et al.* (2024)	OR814155, OR814156	–	–
Derogenidae					
Derogenes varicus	*Hippoglossoides platessoides*	Olson *et al.* (2003)	AY222189	–	–
Genarchella pichileufuensis	*Hatcheria macraei*	Tsuchida *et al.* (2021b)	–	–	LC630952–LC630954
Sclerodistimidae					
Prosogonotrema bilabiatum	*Rhomboplites aurorubens*	Claxton *et al.* (2017)	KU527431	–	–
Gonocercidae					
Gonocerca muraenolepisi	*Muraenolepis marmorata*	Sokolov *et al.* (2016)	LN865026	–	–
Gonocreca oshoro	*Albatrossia pectoralis*	Sokolov *et al.* (2017)	KY197014	–	–
Azygiidae					
Azygia hwangtsiyui	*Ophiocephalus argus*	Wu *et al.* (2020)	–	–	MN844889
Azygia robusta	*Hucho taimen*	Atopkin *et al.* (2023)	–	***	OR350239

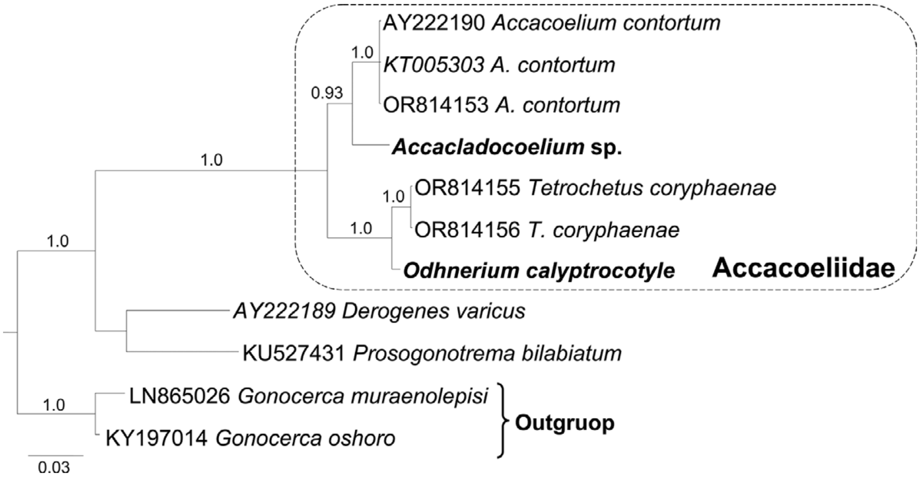

Figure 2. Phylogenetic relationships of *Accacladocoelium sp.* and *Odhnerium calyptrocotyle* ex *Mola mola* off Iturup Island based on partial *28S rRNA* gene sequences, generated with a Bayesian inference algorithm. Posterior probability values lower than 0.9 are not shown. Newly obtained sequences are in bold.

Accacoeliidae clade was subdivided into two groups. The first group contained *Accacoelium contorum* (Rudolphi, 1819), and its sister branch was represented by *Accacladocoelium* sp. The second group included two sister taxa, *Tetrochetus coryphaenae* Yamaguti, 1934, and *O. calyptrocotyle*. *Accacladocoelium* sp. and *O. calyptrocotyle* differed from other molecularly analysed representatives of the Accacoeliidae by 3.24±0.56%–6.6±0.88% and 5.52±0.4%–13.2±1.17%, respectively.

The phylogenetic tree based on nucleotide sequences from a mitochondrial *cox1* gene fragment (Figure 3) included *Accacladocoelium nigroflavum* (Rudolphi, 1819), *Accacladocoelium macrocotyle*

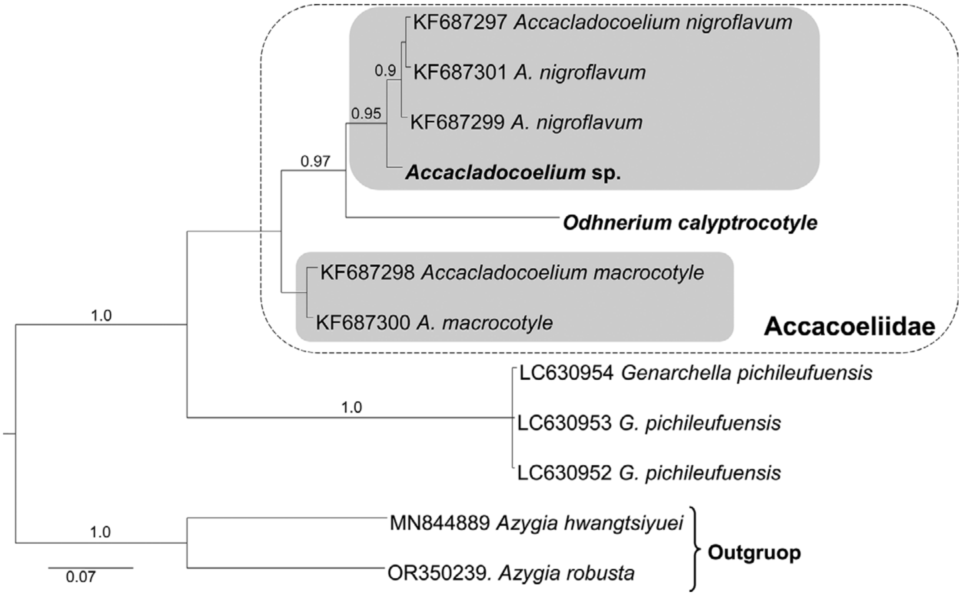

Figure 3. Phylogenetic relationships of *Accacladocoelium sp.* and *Odhnerium calyptrocotyle* ex *Mola mola* off Iturup Island based on partial *cox1* gene sequences, generated with a Bayesian inference algorithm. Posterior probability values lower than 0.9 are not shown. Newly obtained sequences are in bold. Members of the paraphyletic genus *Accacladocoelium* are marked with a grey background.

(Diesing, 1858), *Accacladocoelium* sp., and *O. calyptrocotyle.* The family Accacoeliidae with this species content has poorly supported monophyly, and the genus *Accacladocoelium* Odhner, 1928, was paraphyletic. *Accacladocoelium* sp. was closely related to *A. nigroflavum*, and *O. calyptrocotyle* was moderately supported as sister to this species clade. However, *A. macrocotyle* appeared as an early divergent lineage within Accacoeliidae. The genetic p-distance value between *Accacladocoelium* sp. and *A. nigroflavum* ranged from 2.3±0.75% to 2.56±0.8%, and between *Accacladocoelium* sp. and *A. macrocotyle* – from 9.46±1.42% to 9.72±1.43%. The mean value of p-distance between *O. calyptrocotyle* and *Accacladocoelium* spp. was 15.4±1.82%.

Results of phylogenetic analysis based on the concatenated ribosomal ITS2 and mitochondrial *cox1* gene sequence dataset showed highly supported monophyly for Accacoeliidae and confirmed close relationships between *Accacladocoelium* sp. and *A. nigroflavum* (Figure 4). *Odhnerium calyptrocotyle* and *A. macrocotyle* occupied on this tree a position identical to that on *the cox1 tree* and render the genus *Accacladocoelium* paraphyletic as well.

The poor capacity of the 312 bp fragment of the ITS2 rDNA sequence dataset was not enough to carry out an adequate phylogenetic analysis. For this reason, we provide only p-distance values between different *Accacladocoelium* species based on this molecular marker that were from 2.95±0.94% to 4.25±1.14% between *Accacladocoelium* sp. and *A. nigroflavum* and 3.92±1.09% between *Accacladocoelium* sp. and A. *macrocotyle.* The range of p-distance values between *Accacladocoelium* sp. and *A. nigroflavum* observed because of one of three sequences of the last species from the study of Ahuir-Baraja *et al.* (2015a) under accession no. KF687304 differs from the other two sequences in four substitutions, representing a 1.31±0.63% differentiation value, which was unmentioned by the authors of that study. For this reason, specimens of *Accacladocoelium* sp. from our study and *A. nigroflavum* accession no. KF687304 show greater divergence from each other.

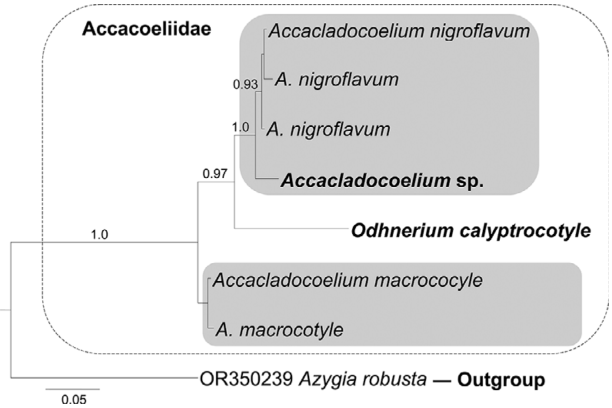

Figure 4. Phylogenetic relationships of *Accacladocoelium sp.* and *Odhnerium calyptrocotyle* ex *Mola mola* off Iturup Island based on concatenated sequences of ITS2 region and *cox1* gene, generated with a Bayesian inference algorithm. Posterior probability values lower than 0.9 are not shown. Newly obtained sequences are in bold. Members of the paraphyletic genus *Accacladocoelium* are marked with a grey background.

Discussion

The results of phylogenetic analysis show that *Accacladocoelium* sp. appears as a distinct lineage closely related to *A. nigroflavum.* The values of p-distances based on mitochondrial *cox1* gene sequences between these digeneans are in the interval between the levels of obvious intra- (<2%) and interspecific (>10%) divergence recorded in accacladocoeliids and derogenids related to it (Ahuir-Baraja et al. 2015a; Bouguerche et al. 2023; Gharbi *et al.* 2024; Tsuchida et al. 2021a). However, p-distance values calculated based on the ITS2 rDNA sequence dataset between *Accacladocoelium* sp. and *A. nigroflavum* are consistent with the level of interspecific differentiation observed in accacladocoeliids and derogenids (Ahuir-Baraja et al. 2015a; Gharbi *et al.* 2024). For this reason, we suggest that *Accacladocoelium* sp. is a distinct species that is close to *A. nigroflavum* genetically.

The genus *Accacladocoelium* Odhner, 1928, is represented by four nominal species (Bray and Gibson 1977), three of which were recorded in the Pacific Ocean: *A. macrocotyle* (Fernández et al. 2016; Luque and Oliva 1993; Pratt and McCauley 1961; Villalba and Fernández 1985), *Accacladocoelium alveolatum* Robinson, 1934 (Luque et al. 2016), and *Accacladocoelium petasiporum* Odhner, 1928 (Zhang et al. 2016). However, the data on the occurrence of *A. petasiporum* in the Pacific Ocean are questionable since the morphology of the ventral sucker in specimens found by Zhang et al. (2016) does not correspond to that of this species as described by Bray and Gibson (1977). Our specimen of *Accacladocoelium* sp. had a non-specialised ventral sucker and is thus clearly not conspecific with *A. petasiporum.* According to the results of our phylogenetic analyses, *Accacladocoelium* sp. also is not conspecific with *A. macrocotyle.* Species affiliation of *Accacladocoelium* sp. needs clarification.

In the present study, we provide the first molecular data for the genus *Odhnerium.* This taxon is closely related to the genus *Tetrochetus* Looss, 1912, in the *28S rRNA* gene-based phylogenetic tree. Since the species of *Tetrochetus*, unlike those of *Accacladocoelium* and *Odhnerium*, are not specific parasites of fish of the family Molidae (e.g., Bray and Gibson 1977), we have not received evidence of monophyly of either *Mola*- or Molidae-associated accacoeliids at present. Results of phylogenetic analysis based on both the mitochondrial *cox1* gene and concatenated ribosomal ITS2 and *cox1* genes of mtDNA show that the genus *Odhnerium* is close to *A. nigroflavum* and a new species, *Accacladocoelium* sp., thereby creating paraphyly of the genus *Accacladocoelium.* We cannot make definitive statements concerning this paraphyly because of the poor molecular data available on accacoeliid species to date. Moreover, monophyly of the Accacoeliidae with paraphyletic *Accacladocoelium* is not supported statistically in the *cox1* gene-based phylogenetic tree, suggesting that other tree topologies of the Accacoeliidae may emerge when additional molecular data on members of this family are included.

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Competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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