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Molecular Analysis of *Pomphorhynchus kashmirensis* based on 18S rDNA and ITS-rDNA

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Abstract

Pomphorhynchus kashmirensis Kaw, 1941 was redescribed to correct inadequacies in the original description. We provide a molecular characterization to qualify its now completed morphological description and to compare with related taxa. Sequences of the 18S rDNA and ITS-rDNA datasets of specimens of *P. kashmirensis* were generated for assessment of gene diversity and the phylogenetic analyses. Comparative sequence analysis indicated interspecific variation between *P. kashmirensis* with different species of *Pomphorhynchus* was 0.2-1.4% and 2.4-36.4% based on 18S rDNA and ITS-rDNA datasets, respectively. The ITS-rDNA region is more variable than 18S rDNA within members of the family Pomphorhynchidae and it appears appropriate for assessment of their biodiversity. Our phylogenetic analyses showed that taxonomic position of the species *P. kashmirensis* is closely related with *P. tereticollis* and *P. laevis*. Also, the systematic status of *Tenuiproboscis* and *Longicollum* is uncertain within the family Pomphorhynchidae. Therefore, further molecular investigations will be needed for better understanding of the phylogenetic relationships in this family.

Keywords: *Pomphorhynchus kashmirensis*; 18S rDNA; ITS-rDNA; Phylogeny

Introduction

Pomphorhynchus kashmirensis Kaw, 1941 is one of 9 species of *Pomphorhynchus* Monticelli, 1905 known from the Jammu-Kashmir regions of the Northern Indian Subcontinent. The original description from *Triplophysa kashmirensis* Hora, 1922 (Nemacheilidae) was inadequate as much of its morphological features could not be adequately visualized or confirmed in text or in illustrations [1]. Only one taxonomic treatment by Fotedar and Dhar was reported since its original description and before the redescription from specimens obtained from *Schizothorax plagiostomus* Heckel, 1838 by Amin et al. In the redescription, comparisons with the Kaw and Fotedar and Dhar accounts were made and 12 SEM images were added presenting new features not previously provided [2,3]. This study complements the morphological aspects of *P. kashmirensis* as redescribed by providing its molecular profile for the first time based on of the 18S rDNA and ITS-rDNA datasets. Furthermore, its phylogenetic relationships with other species of the genus *Pomphorhynchus* and other members of Pomphorhynchidae are analyzed and discussed.

Materials and Methods

DNA Extraction and PCR Amplification

The *P. kasmirensis* material was collected from *Schizothorax plagiostomus* in the Jammu-Kashmir regions in the Northern Indian subcontinent. For molecular analysis, the

genomic DNA was extracted from ethanol-preserved worms (n=3) using Qiagen DNeasy tissue kit (Qiagen Inc., Valencia, California, USA) according to manufacturer's instructions. Finally, purified DNA was kept at – 20 °C until use.

The PCR mixture was performed in a final volume 30 µL containing 15 µL of 2X PCR premix with 1.5 mM MgCl2 (Ampliqon, Denmark), 20 ρM of each primer and 2 µL of the extracted DNA. The partial 18S region was amplified using the forward primer (5′-AGATTAAGCCATGCATGCGTAAG-3′) [4] and reverse primer (5′- ACCCACCGAATCAAGAAAGAG-3′) [5]. PCR amplification of the ITS1-5.8S-ITS2 ribosomal RNA gene was performed using the primers BD1 (5′ GTCGTAACAAGGTTTCCGTA-3′) and BD2 (5-TATGCTTAAATTCAGCGGGT-3′) [6].

The temperature profile for the 18S rRNA gene included an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 60 s, followed by a final extension step at 72°C for 7 min. The PCR conditions of ITS-rDNA region amplification consisted of initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s, followed by a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized with UV transluminator (Vilber Lourmat, Collégien, France). Finally, PCR products were sequenced by an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

The sequence results were edited and trimmed by Chromas software v.2.01 (Technelysium Pty Ltd., Brisbane, Queensland, Australia). Edited sequences were compared with GenBank submitted sequences using the Basic Local Alignment Search Tool (BLAST) [7]. The sequences of 18S rRNA and ITS-rDNA were deposited in GenBank (Accession Numbers: MZ381411-MZ381413 for 18S rRNA and MZ381446-MZ381448 for ITS-rDNA).

Phylogenetic Analysis

Phylogenetic trees were constructed using Maximum-likelihood algorithm and tamura-3- parameter model in MEGA6 software [8]. Also, genetic distances were calculated with p-distance model. The bootstrap value with 1000 replications were used for evaluation of reliability of the tree. The sequences used for the phylogenetic analysis are listed in Tables 1 & 2.

Species	Host	Location	GenBank accession no.	Reference
Pomphorhynchus kashmirensis (Kaw, 1941)	Schizothorax plagiostomus	India	MZ381411-MZ381413	This study
Pomphorhynchus tereticollis (Rudolphi, 1809)	Gammarus pulex	France	AY423347	[9]
Pomphorhynchus laevis (Zoega in Müller, 1776)	Squalius cephalus	Croatia	KF559309	[10]
Pomphorhynchus laevis (Zoega in Müller, 1776)	Rutilus rutilus	Germany	JX014223	[11]
Pomphorhynchus zhoushanensis (Li, Chen, Amin & Yang, 2017)	*Oplegnathus fasciatus* Temminck & Schlegel	China	KY490049-59	[12]
Longicollum pagrosomi (Yamaguti, 1935)	*Oplegnathus fasciatus* Temminck & Schlegel	China	KY490052	[12]
Longicollum pagrosomi (Yamaguti, 1935)	Red sea bream	South Korea	KX641270	[13]
Rhadinorhynchus lintoni (Cable & Linderoth, 1963)	*Selar crumenophthalmus*	USA	JX014224	[11]
Rhadinorhynchus pristis (Rudolphi, 1802)	*Gempylus serpens*	Indonesia	JX014226	[11]
Echinorhynchus borealis (Linstow, 1901)	*Perca fluviatilis*	Finland	MW172281	[14]
Echinorhynchus truttae (Schrank, 1788)	*Thymallus thymallus*	Na	AY830156	[15]
Echinorhynchus gadi (Zoega in Müller, 1776)	*Macrourus berglax*	Greenland	JX014222	[11]
Echinorhynchus gymnocyprii (Liu, Wang & Yang, 1981)	*Ptychobarbus kaznakovi*	China	MT162051	Lei et al. (unpublished)
Echinorhynchus gymnocyprii (Liu, Wang & Yang, 1981)	*Gymnocypris przewalskii*	China	MT162047	Lei et al. (unpublished)

Acanthocephaloides propinquus (Dujardin, 1845)	*Gobius bucchichii*	Na	AY830149	[15]
Acanthocephalus lucii (Müller, 1776)	*Perca fluviatilis*	Na	AY830152	[15]
Acanthocephalus clavula (Dujardin, 1845)	*Anguilla anguilla*	Ireland	MW172278	[14]
Acanthocephalus rhinensis (Amin, Thielen, Münderl, Taraschewski & Sures, 2008)	*Anguilla anguilla*	Germany	MW172279	[14]
Acanthocephalus dirus (Van Cleave, 1931)	*Asellus aquaticus*	Na	AY830151	[15]
Acanthocephalus anguillae (Müller, 1780)	*Asellus aquaticus*	Slovenia	MN394415	[16]
Serrasentis sagittifer (Linton, 1889)	*Lethrinus laticaudis*	Australia	MF426939	[17]
Gorgorhynchoides bullock (Cable & Mafarachisi, 1970)	*Eugerres plumiere*	Na	AY830154	[15]
Outgroup				
Oligacanthorhynchus tortuosa (Leidy, 1850)	Didelphis virginiana	Na	AF064817	[18]

Table 1: Acanthocephalan species represented in the phylogenetic analysis with their host species, locations, GenBank accession numbers, and references based on 18S rDNA gene.

Species	Host	Location	GenBank accession no.	Reference
Pomphorhynchus kashmirensis (Kaw, 1941)	*Schizothorax plagiostomus*	India	MZ381446-MZ381448	This study
Pomphorhynchus tereticollis (Rudolphi, 1809)	*Platichthys flesus*	Germany	JF706705	[19]
Pomphorhynchus tereticollis (Rudolphi, 1809)	*Salmonids*	Germany	MT216144	[20]
Pomphorhynchus laevis (Zoega in Müller, 1776)	*Phoxinus phoxinus*	Slovakia	AY135413	[21]
Pomphorhynchus bosniacus (Kiskároly & Čanković, 1967)	*Alburnus alburnus*	Bosnia and Herzegovina	MK157039	[22]
Pomphorhynchus bosniacus (Kiskároly & Čanković, 1967)	*Barbus barbus*	Bosnia and Herzegovina	MH319901	[22]
Tenuiproboscis keralensis (Kaur, Shamal, Chandran, Binesh, Gishnu, Asokan & Sanil, 2017)	*Scatophagus argus*	India	KU726600 and KU726601	[23]
Pomphorhynchus lucyi (Williams & Rogers, 1984)	*Micropterus salmonoides*	USA	AY135418	[21]
Echinorhynchus gymnocyprii (Liu, Wang & Yang, 1981)	*Ptychobarbus kaznakovi*	China	MT162085	[24]
Echinorhynchus gadi (Zoega in Müller, 1776)	*Gadus morhua morhua*	Barents Sea	EF107648	[25]
Echinorhynchus truttae (Schrank, 1788)	*Na*	Germany	MT216137	[20]
Acanthocephalus nanus (Van Cleave, 1925)	*Cynops pyrrhogaster*	Japan	LC100043	[26]
Acanthocephalus anguillae (Müller, 1780)	*Proteus anguinus*	Slovenia	MN394425	[16]
Rhadinorhynchus dorsoventrospinosus (Amin, Heckmann & Nguyen Van Ha, 2011)	*Decapterus kurroides*	Vietnam	MH384822	[27]
Neoechinorhynchus bullocki (Doolin & Reyda, 2018)	*Catostomus commersonii*	USA	MK017784	[28]
Floridosentis mugilis (Machado Filho, 1951)	Mugil sp.	Gulf of Mexico	KC004179	[29]

Table 2: Acanthocephalan species represented in the phylogenetic analysis with their host species, locations, GenBank accession numbers, and references based on ITS-rDNA region.

Results

An approximately 1225 bp fragment of 18S rDNA gene and a 805 bp fragment of ITS-rDNA region were successfully amplified from the specimens of *P. kashmirensis*. Based on both datasets, there was no intraspecific genetic divergence found among the obtained isolates of *P. kashmirensis*. The 18S rDNA dataset (1144 nt) included 20 sequences of species representing five families (*Pomphorhynchidae, Rhadinorhynchidae, Echinorhynchidae* and *Arhythmacanthidae*) within the Echinorhynchida and the novel sequences of *P. kashmirensis*. The ITS-rDNA dataset (747 nt) included 13 sequences of species of four families (*Pomphorhynchidae, Rhadinorhynchidae* and *Echinorhynchidae*) of Echinorhynchida and the new sequences of *P. kashmirensis*.

The nucleotide divergence was noted between *P. kashmirensis* with *P. laevis* (KF559309 and JX014223), *P. zhoushanensis* (KY490049 and KY490050), *P. tereticollis* (AY423347), *Longicollum pagrosomi* (KY490052 and KX641270), *Rhadinorhynchus lintoni* (JX014224), *R. pristis* (JX014226), *Acanthocephaloides propinquus* (AY830149), *Serrasentis sagittifer* (MF426939), *Gorgorhynchoides bullocki* (AY830154), *Echinorhynchus* spp. and different species *Acanthocephalus* based on partial 18S rDNA sequence were 0.2-0.3%, 1.4%, 0.2%, 1.4%, 0.8%, 0.6%, 7.6%, 11.5%, 11.6%, 2.1-3.9% and 7.2-7.5%, respectively. Inter-generic differences between *P. kashmirensis* with *P. tereticollis* (JF706705 and MT216144), *P. laevis* (AY135413), *P. bosniacus* (MK157039 and MH319901), *P. lucyi* (AY135418), *Tenuiproboscis keralensis* (KU726600 and KU726601), *Rhadinorhynchus dorsoventrospinosus* (MH384822), *Echinorhynchus* spp. and *Acanthocephalus* spp. based on partial ITS-rDNA ITS-rDNA region were 2.5%, 2.4%, 5.5%, 36.4%, 15.5-15.6%, 49.2%, 36.3-38.2% and 49.6-50.1%, respectively.

The phylogenetic reconstruction based on the partial sequence spanning the 18S rDNA indicated that our sequences of *P. kashmirensis* (MZ381411-MZ381413) grouped with *P. laevis* (KF559309 and JX014223), *P. tereticollis* (AY423347), *R. lintoni* (JX014224) and *R. pristis* (JX014226). Additionally, *P. zhoushanensis* (KY490049 and KY490050) and *L. pagrosomi* (KY490052 and KX641270) appeared as sister group of mentioned clades (Figure 1).

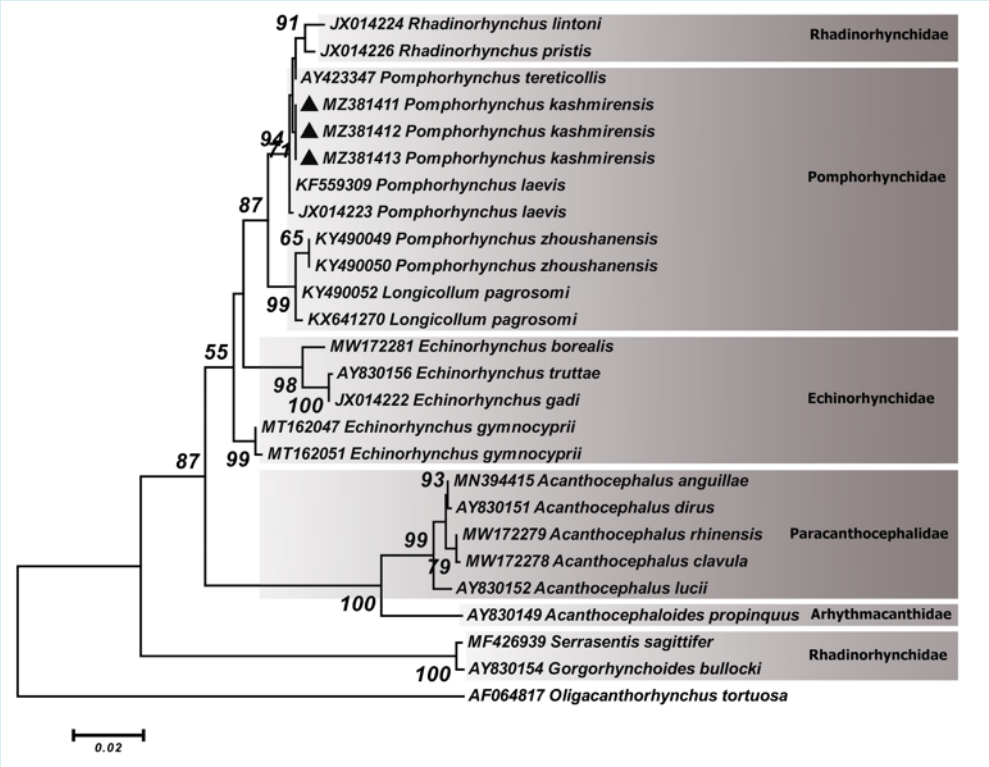

Figure 1: Phylogenetic tree of isolates of the Pomphorhynchus kashmirensis obtained in this study (▲) and other members of the families Pomphorhynchidae, Echinorhynchidae, Arhythmacanthidae and Rhadinorhynchidae retrieved from GenBank based on the partial 18S rDNA gene. The tree was constructed based on the maximum likelihood method and the Tamura 3-parameter model in MEGA6. *Oligacanthorhynchus tortuosa* sequence was used as the out group. Bootstrap values lower than 50 were omitted.

According to phylogenetic analyses based on the ITS-rDNA ITS-rDNA region, our sequences of *P. kashmirensis* (MZ381446-MZ381448) clustered with *P. tereticollis* (JF706705 and MT216144) and *P. laevis* (AY135413) but with marginal support. Also, *P. bosniacus* (MK157039 and MH319901) appeared as sister group of mentioned clades with higher bootstrap value. The sequences of *T. keralensis* (KU726600 and KU726601) as other genus of Pomphorhynchidae family were appeared to be a sister taxon of *P. tereticollis*, *P. laevis* and *P. bosniacus* with 100% of bootstrap support. However, the species of *P. lucyi* (AY135418) was located at the basal position to the members of the clade of Pomphorhynchidae (Figure 2).

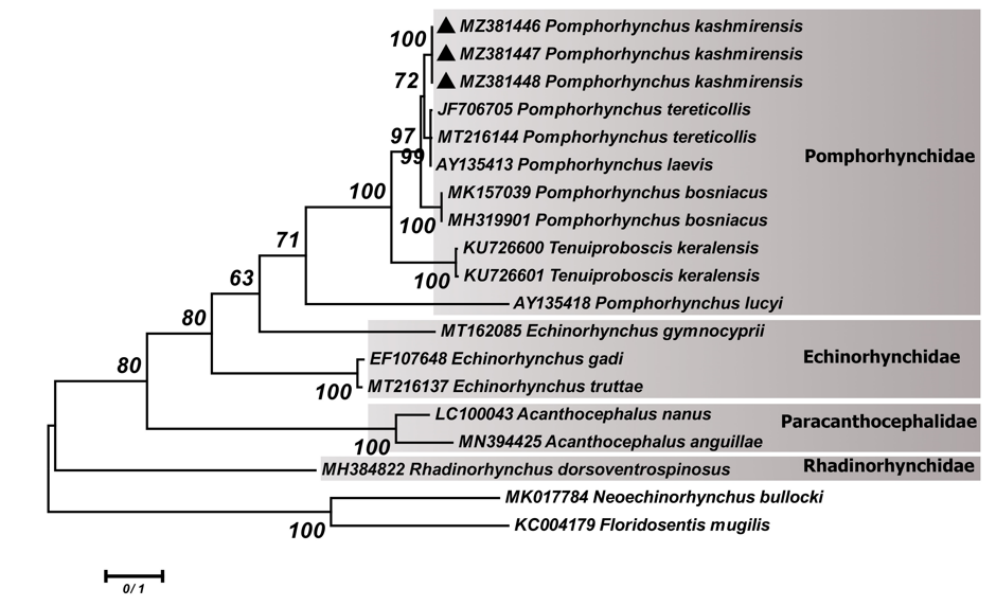

Figure 2: Phylogenetic tree of isolates of the *Pomphorhynchus kashmirensis* obtained in this study (▲) and other members of the families *Pomphorhynchidae, Echinorhynchidae* and *Rhadinorhynchidae* retrieved from GenBank based on the ITS-rDNA region. The tree was constructed based on the maximum likelihood method and the Tamura 3-parameter model in MEGA6. Neoechinorhynchus bullocki and *Floridosentis mugilis* sequences were used as the out group. Bootstrap values lower than 50 were omitted.

Discussion

The genus *Pomphorhynchus* presently contains twenty-nine species which are ordinary parasitic worms within the intestinal tract of freshwater fishes, and sometimes marine fishes and amphibians [30]. Until now, only few species of this genus have been characterized using molecular analysis [12,19,21,31-33]. The validity of *P. kashmirensis* as redescribed by Amin, et al. is confirmed by the presented molecular analysis. Herein, molecular profile of *P. kashmirensis* is obtained based on partial 18S rDNA and ITS-rDNA genes for understanding its taxonomic relationships within the family Pomphorhynchidae and with other families of Echinorhynchida.

The level of interspecific nucleotide variation between *P. kashmirensis* and other species of *Pomphorhynchus* registered in Gen Bank was 0.2-1.4% and 2.4-36.4% based on 18S rDNA and ITS-rDNA sequences, respectively. These results indicated that ITS-rDNA is more variable between the species than 18S rDNA. Based on the partial 18S rDNA, intergeneric variation between *P. kashmirensis* and *L. pagrosomi* another genus of the family Pomphorhynchidae, was 1.4%. Also, based on partial ITS sequences, inter-generic variations between *P. kashmirensis* and *T. keralensis* sequences another genus of Pomphorhynchidae family was 15.5-15.6%. Similar to our study, other researchers claimed ITS rDNA sequences has high level of interspecific nucleotide variation in different species of *Pomphorhynchus* [21,12]. In addition, some molecular studies on acanthocephalans illustrated that ITS rDNA region is a relatively variable region and has been used as a suitable molecular marker for inferring phylogenetic relationships in some acanthocephalan genera such as *Acanthogyrus* [34], *Corynosoma* [35], *Neoechinorhynchus* [36] and *Leptorhynchoides* [37].

The small subunit 18S rRNA gene is one of the most frequently used molecular markers to determine genetic diversity and phylogenetic relationships among acanthocephalans. This gene is fairly conserved with a slow

evolutionary rate and is usually utilized to infer phylogenetic analysis among the major classes of Acanthocephala [38]. In this study, relatively small genetic variation was found between members of the genus *Pomphorhynchus* based on18S rRNA gene. Also, there were small genetic differences between species of *Pomphorhynchus* and *Rhadinorhynchus* based on this gene. This issue has been investigated and it has been concluded that these two sequences are not really *Rhadinorhynchus*, but actually Pomphorhynchids [39].

The phylogenetic tree of the 18S rDNA gene (Figure 1) showed that the sequences of *P. kashmirensis* (MZ381411-MZ381413) obtained in the current study grouped with *P. laevis* (KF559309 and JX014223) from Croatia and Germany and *P. tereticollis* (AY423347) from France. However, *R. lintoni* (JX014224) and *R. pristis* (JX014226) were nested within this cluster but the genus *Rhadinorhynchus* was located well apart from the members of the family Pomphorhynchidae in the ITS-rDNA tree. Our 18S rDNA phylogenetic tree is similar to a previously published tree [39,40], where the genus *Rhadinorhynchus* was found to be clustered with these species of *Pomphorhynchus*. In addition, *P. zhoushanensis* (KY490049 and KY490050) from China and *L. pagrosomi* (KY490052 and KX641270) from China and Korea were grouped together in one cluster and appeared as a sister group of the mentioned clade with high statistical support. Some authors have also observed a similar situation in *L. pagrosomi* in *Pomphorhynchus* clade [12,40].

Similar to the 18S rDNA tree, the phylogenetic tree of ITS-rDNA (Figure 2) also illustrated that the *P. kashmirensis* has a close relationship with *P. tereticollis* (JF706705 and MT216144) from Germany and *P. laevis* (AY135413) from Slovakia. Additionally, *P. bosniacus* isolates (MK157039 and MH319901) from Bosnia and Herzegovina clustered as a sister group of the mentioned clade with high statistical support. Similar to our study, Li, et al. showed that the genus *Tenuiproboscis* was nested within the core of *Pomphorhynchus* in the phylogenetic trees [12]. Meanwhile, the species of *P. lucyi* (AY135418) isolated from USA was placed separately from other members of genus *Pomphorhynchus.* In the current study, the higher level of variation in ITS-rDNA region compared to the 18S rDNA gene improved resolution of the relationships within the family *Pomphorhynchidae* with high bootstrap support.

Similar to our findings, Li et al. proposed that genus *Pomphorhynchus* is a polyphyletic taxon based on three different genetic markers (18S rDNA, ITS rDNA and *Cox1*). It is because members of the *Pomphorhynchus* were mixed with the genera *Longicollum* and *Tenuiproboscis* [12]. The current phylogenetic position of *Longicollum* and *Tenuiproboscis* remains questionable and more investigations required to elucidate the phylogenetic relationship between these genera.

Conclusion

The present phylogenetic analyses based on both 18S rDNA and ITS-rDNA sequence data showed the taxonomic position of *P. kashmirensis* being closely related with *P. tereticollis* and *P. laevis*. The ITS-rDNA sequences of members of genus *Pomphorhynchus* have more variation than 18S rDNA which it can be useful marker for achieving a proper assessment of gene diversity and phylogenetic analyses. In addition, the systematic status of *Tenuiproboscis* and *Longicollum* is uncertain within the Pomphorhynchidae. Therefore, obtaining sequence data from other members of the family Pomphorhynchidae and providing additional genetic markers would be useful for better understanding of the phylogenetic relationships in this family.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare no conflict of interest and compliance with all relevant ethical standards.

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