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Genetic diversity of the major Dengue vectors *Aedes aegypti* and *Aedes albopictus* based on mitochondrial COI gene

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Abstract

Dengue fever has emerged as a significant public health crisis in Bangladesh transmitted by *Aedes* mosquitoes. With a sharp rise in cases and fatalities, it poses a serious health risk to the communities. An authentic identification of major species of dengue vector is an utmost necessity for the control of dengue endemic. This study aims to understand molecular characterization and phylogenetic relationship of the key dengue vector mosquitoes. Specimens were collected from the wild habitat of Dhaka city and identified through morphological and molecular approaches. The Cytochrome c oxidase subunit I (COI) genes were amplified from the extracted genomic DNA by polymerase chain reaction using a pair of universal primers. The target nucleotide sequences of COI gene of collected mosquitoes were confirmed two species *viz. Aedes aegypti* and *Ae. albopictus*. Both species exhibit an A+T bias, with A+T content significantly higher (65.82% and 66.89%, respectively) compared to G+C content (34.18% and 33.11%, respectively). The genetic distance between the two species was found as 10.7. In the multiple sequence alignment, a total of 73 polymorphic sites were identified which can be distinguished from one another. Comparative analysis found moderate to high evolutionary divergence among the species from five different continents. Maximum likelihood tree indicates that members of *Ae. aegypti and Ae. albopictus* formed two separate clusters that supports our findings in evolutionary divergence. Further research needed on the remaining Dengue vector mosquitoes' phylogeny, comparative genomic analysis and innovative vector control strategies.

Introduction

The public's health is greatly impacted by *Aedes* mosquitoes, as they spread several viral disease serotypes that cause dengue, yellow fever, and chikungunya in humans[1,2]. Bangladesh has experienced sporadic outbreaks of dengue since 1964, followed by the first large outbreak in 2000[3]. The number of cases and deaths surged during outbreaks in 2019 and 2023, with > 100,000 and > 250,000 infected individuals and > 160 and > 1,700 deaths, respectively[4,5]. Controlling the mosquito population is essential for illness prevention

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because no vaccine or antiviral medication is currently available. In this regard, knowledge of mosquito genetic structure and bionomics is crucial when dealing with sensitive or refractory vector species. For identification and vector competency studies, different *Aedes* species have been mass-reared in laboratories over the last few decades. Conventionally, the morphological traits of mosquitoes are used for identification[6]. However, only 10% of the world's species have been identified to date due to the labor-intensive and specialized nature of the process[2,7].

The traditional taxonomic method for mosquito identification, which is based on morphological characteristics, is time-consuming, requires specialized expertise, and can be problematic when dealing with damaged specimens[8]. However, the use of specific molecular markers is a more reliable and faster alternative for vector identification. Understanding the dynamics of vector-borne diseases and achieving taxonomic objectives both depend on the routine characterization of mosquito populations and the updating of diagnostic criteria[9].

Accurate and precise identification of mosquitoes is essential for developing effective mosquito control strategies. Molecular techniques are the most reliable methods to ensure accurate identification. DNA barcoding is a taxonomic method whose goal is not to determine patterns of relationship but to identify an unknown sample within a preexisting classification system[10]. In this context, the study of evolutionary relationships and species identification heavily relies on mitochondrial DNA (mtDNA). Among various mitochondrial genes, the cytochrome c oxidase subunit I (COI) gene is one of the most informative sections used in molecular phylogeny, genetic diversity studies, and species identification in recent mosquito research[11–13]. Only a few studies have been done on the molecular characterization of major vector mosquitoes in Bangladesh[14,15].

The current study aims to construct a DNA reference dataset employing COI genes from principal dengue vectors in Bangladesh and to analyze their genetic diversity and comparative genomics across different mosquito species reported from various parts of the world.

Materials and Methods

Mosquito sampling and morphological identification: Larval mosquitoes were collected during August and November 2023 from Dhaka. The captured larvae were raised to adulthood in insect rearing cage in entomology laboratory, Department of Zoology, University of Dhaka. A stereo zoom microscope was used to identify the adult mosquitoes that emerged from each collection in order to separate them into different genera and species. Dichotomous taxonomic keys were used to determine the species and sex provided by Huang[16] and Rueda[17]. Samples were preserved in -20°C individually.

*Genomic DNA extraction, amplification by PCR and sequencing***:** DNA extraction kit (Qiagen® Dneasy Blood & Tissue Kits (USA)) was used to extract DNA from a single

mosquito sample. Three biological replicates were considered per species. The quality and concentration of extracted DNA was checked in 1% agarose gel and spectrophotometer (NanoDrop™ One Microvolume UV–Vis Spectrophotometer, USA). To amplify the partial COI gene, a primer pair LCO-1490 (forward) 5'-TCAACAAATCATAAGGACATTGG-3'and HCO-2198 (reverse) 5'TAAACTTCAGGGTGTCCAAAGAATCA-3' was used[18]. The PCR reaction volume was 25µl, containing 23 µl of PCR Master Mix (12.5 µl Taq Polymerase, 8.5 µl Nano Pure water, 1 µl of both forward and reverse primer) and 2 µl of DNA sample and mixed properly. The PCR amplifications were performed following the conditions: initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 1 min (denaturation), 45.5°C for 1 min (annellation), 72°C for 2 min (extension), followed by a final extension at 72°C for 7 min. The PCR products were checked in 1% agarose gel and purified using PCR purification kit (PureLink™ PCR purification kit,). The purified PCR products were sent to First BASE laboratories, Malaysia for capillary sequencing (Sanger sequencing) at both directions.

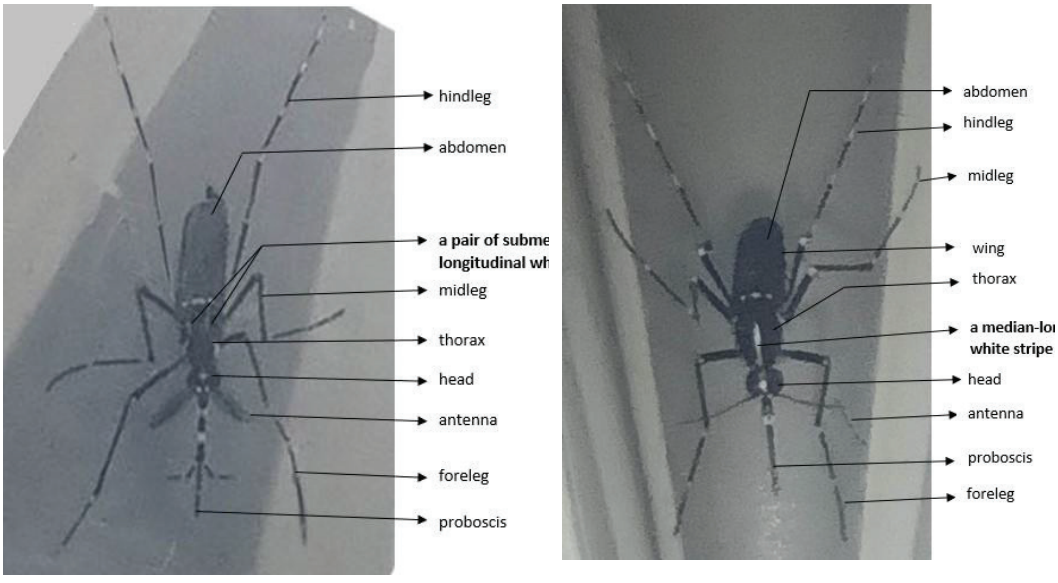
Bioinformatics analysis: The generated .abi file sequences were checked manually and assembled using CAP3[19]. A nucleotide BLAST search with similarity cut off of ≥ 99% was performed at NCBI GenBank to confirm species identity and deposited. Nucleotide compositions of generated sequences were calculated in MEGA 11 v11 software. For comparative analysis, partial COI gene sequences of respective mosquito species from the NCBI GenBank database were downloaded. The sequences were aligned using MUSCLE[20] in ME 1, and the sequences were trimmed to keep the similar regions across all sequences with Seaview5 software[21]. The genetic distance was calculated using MEGA 11[22]. Polymorphic sites were identified by DnaSP v6[23]. Haplotype analysis was conducted using Popart 1.7[24] based on the TCS network. Subsequently, Maximum Likelihood (ML), Neighbor-Joining (NJ) and Maximum-Parsimony (MP) phylogenetic trees were constructed using Mega 11, with bootstrap analysis with 1000 replicates and the trees were visualized using iTOL v5[25]. *Culex quinquefasciatus* was used as outgroup.

Table 1. List of GenBank-retrieved sequences and their respective countries used in the analysis

Sl. No.	Species	Country	GenBank Accession No.	Sl. No.	Species	Country	GenBank Accession No.
1	*Aedes aegypti*	France	HQ688297.1	8	*Aedes albopictus*	UK	MK505609.1
2	*Ae. aegypti*	Kenya	MK300224.1	9	*Ae. albopictus*	Australia	KU495082.1
3	*Ae. aegypti*	Australia	OK285183.1	10	*Ae. albopictus*	China	OR965075.1
4	*Ae. aegypti*	Congo	MN298998.1	11	*Ae. albopictus*	Brazil	OQ983540.1
5	*Ae. aegypti*	Brazil	KU936158.1	12	*Ae. albopictus*	Congo	PP902485.1
6	*Ae. aegypti*	Mexico	OQ338194.1	13	*Ae. albopictus*	India	PP892778.1
7	*Culex quinquefasciatus*	UK	MK505609.1	14	*Ae. albopictus*	USA	PP108371.1

Results and Discussion

Morphological identification: Our morphological identification resulted in the identification of two distinct *Aedes* species, namely *Ae. aegypti* and *Ae. albopictus*. *Ae. aegypti* showed its characteristic brown scutum with a pair of submedian-longitudinal white stripes and mesepimeron with two well separated white scale patches (Fig. 1. a). The anterior portion of mid femur with a longitudinal white stripe, and clypeus with white scale patches. On the other hand, the scutum of *Ae. albopictus* showed a narrow median-longitudinal white strip (Fig. 1. b) and mesepimeron with white scale patches (not separated), forming V-shaped white patch present. These distinct features confirm the identities of respective species. But often, morphological identification can be erroneous, due to cryptic species or phenotypic plasticity and this can result in misidentification. For proper identification, mitochondrial COI gene was sequenced.



a b

Fig. 1. Dorsal view of the current study species (a) *Aedes aegypti* and (b) *Aedes albopictus*.

Amplification of COI gene: To verify the desired fragment of COI gene has been amplified successfully, a PCR was performed using universal COI primer and the quality was checked in 1% agarose gel electrophoresis. The gel documentation image showed that all the PCR products showed clear bands between 500 and 700bp of DNA ladder (Fig. 2), which is congruent with the expected product sizes. It reveals that desired COI gene of mtDNA were properly polymerized. The visualized PCR product contained no double bands on agarose gel, thus indicating that sequences obtained were targeted mitochondrial DNA and not nuclear or mitochondrial mistargets.

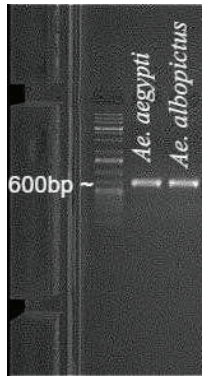


Fig. 2. Agarose gel electrophoresis of mitochondrial Cytochrome c oxidase subunit I (COI) from *Ae. aegypti* and *Ae. albopictus*. L = 100 bp Ladder.

Molecular identification and comparative genomics: After quality trimming, we found sequences spanning 670 bp and 598bp in *Ae. aegypti* and *Ae albopictus*, respectively. The sequenced genes were compared with the available sequences of the NCBI's GenBank and the result of BLAST showed 99.10 to 100% homology to the same species from different geographical areas.

A BLAST search of samples, morphologically identified as *Ae. aegypti*, revealed 99.10 to 99.85% homology with the *Ae. aegypti* COI gene sequences in GenBank, submitted from Congo, Kenya, Mexico and France, with >99% query covered (Table 2). It indicates that the observed sample is *Ae. aegypti*. Another BLAST analysis with morphologically identified *Ae. albopictus* sample, showed 100% homology with the pre-existing sequence PP902485.1, PP892778.1, PP108371.1 and OR965075.1 of the database sequences from Congo, India, USA and China, respectively (Table 2). It proved that the observed species is *Ae. albopictus*.

The obtained two sequences of mtCOI gene of two major dengue vectors *Ae. aegypti* and *Ae. albopictus* were submitted to NCBI GenBank database with the accession numbers PQ014146 and PQ180352.

Table 2. BLAST analysis for *Aedes Aegypti* and *Aedes albopictus*

Species	Total score	Query cover (%)	Identity (%)	E value	Country	GenBank Acc. No.
Aedes aegypti	1219	99	99.55	0.0	Congo	MN298998
	1210	100	99.25	0.0	Kenya	MK300224
	1205	97	99.85	0.0	Mexico	OQ338194
	1205	100	99.10	0.0	France	HQ688297
Aedes albopictus	1105	100	100	0.0	Congo	PP902485
	1105	100	100	0.0	India	PP892778
	1105	100	100	0.0	USA	PP108371
	1105	100	100	0.0	China	OR965075

The comparison of the nucleotide composition of the partial COI gene sequence in two major dengue vector species, *Ae. aegypti* and *Ae. albopictus* are shown in Table 3. Both species exhibit an A+T bias, with A+T content significantly higher (65.82% and 66.89%), compared to G+C content (34.18% and 33.11%), consistent with the findings of Rain *et al.*[14]. In *Ae. aegypti*, the percentages of adenine (A), thymine (T), guanine (G), and cytosine (C) are 27.16%, 38.65%, 16.72%, and 17.46%, respectively, across 670 base pairs, resulting in an A+T content of 65.82% and a G+C content of 34.18% which is similar to Rain *et al.*[14]. On the contrary, in *Ae. albopictus*, adenine and thymine content were slightly higher, at 28.43% and 38.46%, while guanine and cytosine content were slightly lower, at 15.89% and 17.22%, respectively, across 598 base pairs, with an A+T content of 66.89% and a G+C content of 33.11%. These findings highlight a slight variation in nucleotide composition between the two species, with *Ae. albopictus* showing a marginally higher A+T percentage. This data is valuable for molecular studies, such as phylogenetic analysis or species differentiation, and reflects the typical A+T bias observed in mitochondrial DNA of insects.

Table 3. Nucleotide composition of COI gene sequence of selected major dengue vectors species

Name of species	%A (Number)	%T (Number)	%G (Number)	%C (Number)	Total bp	A+T	G+C
Aedes aegypti	27.16 (182)	38.65 (259)	16.72 (113)	17.46 (117)	670	65.82	34.18
Aedes albopictus	28.43 (170)	38.46 (223)	15.89 (95)	17.22 (103)	598	66.89	33.11

While comparing the genetic structures of current study with that of few other countries, we found moderate to high evolutionary divergence. The number of base differences per site from averaging over all sequence pairs between groups are shown in Table 4 and 5. While comparing our *Ae. aegypti COI* gene sequence, the lowest genetic distance (0.00) was found with the sequence of Brazil and the highest genetic distance (0.91) was found for Kenya and France (Table 4). For *Ae. albopictus,* no genetic distance (0.00) was found with the sequence of Congo, India, Australia, USA and China. On the contrary, the highest genetic distance (6.27) was found for Brazil which usually found between two species (Table 5). The results indicate, even though COI gene based molecular identification work well for different *Aedes* species, but for population level diversification, it is important to utilize fast evolving nuclear genes, e.g. microsatellites.

Table 4. Estimates of evolutionary divergence over sequence pairs of *Aedes aegypti* of different countries under 6 continents

Aedes aegypti	Australia	Congo	**Present Study (BD)**	Kenya	Mexico	France	Brazil
Australia		0.004	0.004	0.005	0.004	0.005	0.003
Congo	0.72		0.003	0.003	0.000	0.004	0.000
Present Study (BD)	0.72	0.46		0.004	0.002	0.004	0.000
Kenya	0.96	0.46	0.91				0.003
Mexico	0.50	0.00	0.31	0.47			0.000
France	1.20	1.22	0.91	0.76	1.09		0.005
Brazil	0.30	0.00	0.00	0.58	0.00	0.97	

Standard error estimate(s) are shown above the diagonal. BD = Bangladesh.

Table 5. Estimates of evolutionary divergence over sequence pairs of *Aedes albopictus* of different countries under 6 continents

Aedes albopictus	Congo	India	USA	**Present Study (BD)**	UK	Australia	Brazil	China
Congo		0.002	0.000	0.000	0.002	0.002	0.007	0.002
India	0.15		0.002	0.000	0.002	0.002	0.007	0.002
USA	0.00	0.15		0.000	0.002	0.002	0.007	0.002
Present Study (BD)	0.00	0.00	0.00		0.002	0.000	0.007	0.000
UK	0.32	0.32	0.32	0.17		0.002	0.007	0.002
Australia	0.16	0.32	0.16	0.00	0.17		0.007	0.000
Brazil	6.27	6.27	6.27	6.27	6.26	6.27		0.007
China	0.16	0.16	0.16	0.00	0.16	0.00	6.27	

Multiple sequence alignment: To compare the sequences obtained, multiple sequence alignment was performed using Seaview software. A total of 73 polymorphic sites were identified in around 598 bp sequence. The polymorphic sites were shown in table 6. Mitochondrial COI gene can be considered as highly conserved one in maximum organism like fish, insect. Based on the revealed polymorphic sites, these two species can be distinguished from one another.

Table 6. Inter specific polymorphic sites analysis of COI gene sequence of *Ae. aegypti* and *Ae. albopictus*

Serial No.	Polymorphic sites	*Ae. albopictus*	*Ae. aegypti*	Serial No.	Polymorphic sites	*Ae. albopictus*	*Ae. aegypti*	Serial No.	Polymorphic sites	*Ae. albopictus*	*Ae. aegypti*
1	21	A	T	**26**	249	T	A	**51**	393	A	C
2	28	G	A	**27**	252	A	G	**52**	396	T	A
3	40	A	G	**28**	255	A	T	**53**	405	A	G
4	41	T	C	**29**	258	G	T	**54**	429	A	G
5	51	A	C	**30**	261	G	A	**55**	438	A	G
6	54	T	C	**31**	262	C	T	**56**	444	A	G
7	72	A	G	**32**	264	T	A	**57**	445	G	T
8	78	T	C	**33**	267	T	A	**58**	447	T	A
9	99	T	A	**34**	270	T	C	**59**	450	T	G
10	102	T	A	**35**	273	T	A	**60**	457	C	T
11	111	T	A	**36**	285	C	T	**61**	459	T	A
12	126	T	C	**37**	288	A	G	**62**	471	T	C
13	138	A	G	**38**	291	T	A	**63**	480	G	T
14	141	T	A	**39**	297	A	T	**64**	486	A	T
15	144	C	T	**40**	306	G	A	**65**	492	A	T
16	165	C	T	**41**	318	C	T	**66**	504	T	C
17	169	C	T	**42**	321	T	C	**67**	522	A	T
18	174	A	T	**43**	327	T	A	**68**	525	C	T
19	177	C	T	**44**	345	G	A	**69**	528	A	T
20	184	C	T	**45**	351	A	T	**70**	534	C	T
21	207	T	C	**46**	363	A	T	**71**	567	T	C
22	216	A	G	**47**	373	T	C	**72**	582	T	C
23	238	T	C	**48**	375	A	T	**73**	594	T	C
24	243	C	T	**49**	384	G	T				
25	246	C	T	**50**	390	C	T				

Haplotype: The haplotype network analysis of the mitochondrial COI gene (cytochrome oxidase I) in *Aedes* species, including *Ae. albopictus* and *Ae. aegypti*, the major dengue vectors, provides valuable insights into their genetic diversity and evolutionary relationships (Fig. 3). Each black circle represents a unique haplotype (genetic sequence) found in *Aedes* species, with larger circles indicating haplotypes of higher frequency, meaning more sequences share these haplotypes. The blue hatch marks and numbers along the connecting lines represent mutational steps (nucleotide differences) between haplotypes. *Ae. albopictus* and *Ae. aegypti* are separated from their common ancestor by 43 mutational steps. Rain *et al.*[14] found that *Ae. aegypti* and *Ae. albopictus* are separated from their common ancestor by 33 and 38 mutational steps, respectively. The haplotypes of *Ae. aegypti* with accession numbers MN298998.1, OK285183.1, PQ180352.1, and MK300224.1 are closely related and form a smaller cluster, while haplotype KU495082.1 is more distantly related. On the other hand, the haplotypes of *Ae. albopictus* form a large cluster where MK505609.1 separated with 1 mutational step. Shorter connections, such as "(1)" or "(2)," indicate closely related haplotypes with minor genetic differences. This network highlights the complexity of *Aedes* genetics and emphasizes the importance of haplotype analysis in understanding vector biology and designing effective strategies for controlling dengue transmission.

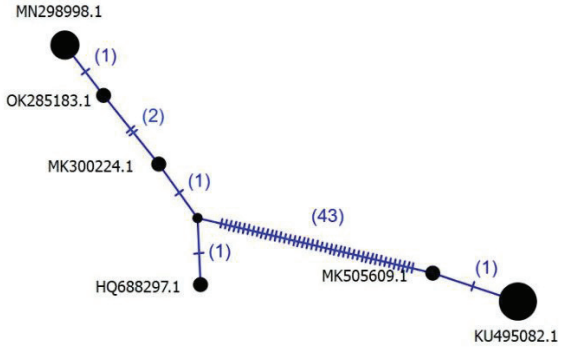


Fig. 3. Haplotype analysis of the mitochondrial COI gene in major dengue vectors (*Aedes* species) constructed using Popart 1.7 based on the TCS network. Black circles represent individual haplotypes, while small black circles denote the common ancestor. Mutational steps are indicated by hatch marks and numbers.

Phylogenetic analysis: Phylogenetic trees were constructed using partial COI sequences in order to identify the phylogenetic position of principal dengue vectors *Ae. aegypti* and *Ae. albopictus* of the present study. A total of 16 sequences (8 *Ae. albopictus*, 7 *Ae. aegypti* and 1 *Culex quinquefasciatus* as out group*)* were taken. Maximum likelihood tree (Fig. 4) indicates that members of *Ae. aegypti and Ae. albopictus* formed two separate clusters. The high bootstrap values of the two clades reveal strong relation among them in both species. The phylogeny showed that, our *Ae. aegypti* sequence showed monophyletic relation of *Ae. aegypti,* reported from Congo, Brazil and Mexico. Interestingly, our *A. albopictus* showed monophyly with Congo, India, Australia, USA and China. The tree supports our findings in evolutionary divergence, where similar low variability was also indicated.

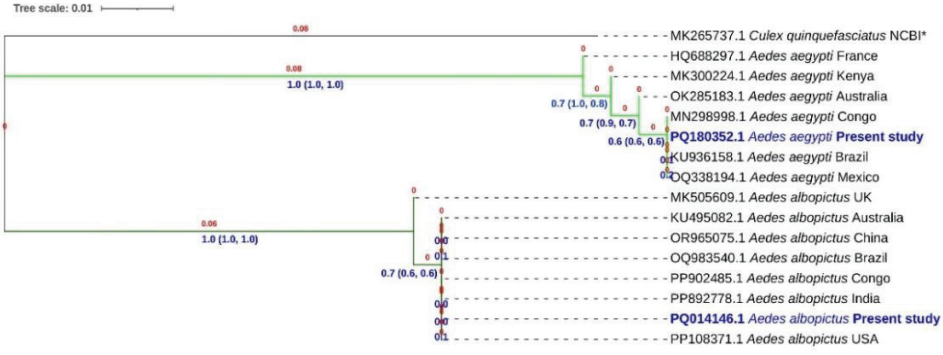


Fig. 4. Molecular phylogenetic analysis of *Aedes* COI gene sequences using ML method in MEGA 11 and visualized by iTOL v5. The branch length value shown in red color. The values in blue color inside parentheses denote the bootstrap values from the MP and NJ methods, respectively. The new sequences generated in the study are indicated with present study and out group as asterisk (*).

Conclusion

The present study involved the generation and submission of mtCOI gene sequences for two species of dengue vector mosquitos to the GenBank database and attempted to identify and characterize *Ae. aegypti* and *Ae. albopictus* at molecular level based on those gene sequences. The study also investigated genetic diversity of the two *Aedes* species from Bangladesh with other countries under five continents. The findings provide a foundation for future research, which is crucial for advancing our understanding of mosquito phylogeny and for informing the development of novel vector control strategies.

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Declaration

The authors declare that they have no conflicts of interest.

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