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The complete chloroplast genome of *Limonium tetragonum* (Plumbaginaceae) isolated in Korea

Yongsung KIM, Hong XI[1] and Jongsun PARK[1]*

Honam National Institute of Biological Resources, Mokpo 58762, Korea
[1]InfoBoss Inc. and InfoBoss Research Center, Seoul 06278, Korea
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ABSTRACT: The chloroplast genome of *Limonium tetragonum* (Thunb.) Bullock, a halophytic species, was sequenced to understand genetic differences based on its geographical distribution. The cp genome of *L. tetragonum* was 154,689 bp long (GC ratio is 37.0%) and has four subregions: 84,572 bp of large single-copy (35.3%) and 12,813 bp of small single-copy (31.5%) regions were separated by 28,562 bp of inverted repeat (40.9%) regions. It contained 128 genes (83 protein-coding genes, eight rRNAs, and 37 tRNAs). Thirty-five single-nucleotide polymorphisms and 33 INDEL regions (88 bp in length) were identified. Maximum-likelihood and Bayesian inference phylogenetic trees showed that *L. tetragonum* formed a sister group with *L. aureum*, which is incongruent with certain previous studies, including a phylogenetic analysis.

Keywords: *Limonium tetragonum*, chloroplast genome, phylogenetic analysis, intraspecific variation, Plumbaginaceae

Limonium, belonging to Plumbaginaceae family, is a cosmopolitan halophytic genus, and a few species inhabit alkaline soil away from the coastal area (Kubitzki, 1993; Morgan and Funnell, 2018). *Limonium tetragonum* (Thunb.) Bullock, distributed in coastal areas of Korea and Japan, is a biennial halophytic species with radical leaves (8–15 × 1.5–3 cm) and yellow corolla (Owhi, 1965; Park, 2007; Lee et al., 2011; Park et al., 2020a). The crude extracts and solvent-partitioned fractions of whole plants of *L. tetragonum* display antioxidant (Lee et al., 2011) and anti-cancer activities (Kong et al., 2008); ethyl acetate soluble fraction of aerial parts exhibits anti-liver fibrosis (Kim et al., 2016); that of whole plants shows anti-alcohol toxicity (Kim et al., 2015); and its methanol extracts of whole plants present hepatoprotective activities (Yang et al., 2014). We sequenced *L. tetragonum* from the western coastal area of Korea for investigating intraspecific variations on chloroplast genomes with chloroplast of *L. tetragonum* isolated in eastern seashore of Korea.

Materials and Methods

Plant material

We collected *L. tetragonum* in Aphaedo island in Shinan-gun, Jeollanam-do, Korea (34.8612N, 126.32629E). A voucher specimen and genomic DNA were deposited in the InfoBoss Cyber Herbarium (IN, the voucher number IB-00899).

DNA extraction and chloroplast genome determination

The total genomic DNA was extracted from fresh leaf by using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Genome sequencing was performed using HiSeqX at Macrogen Inc., Korea, and *de novo* assembly was done by Velvet v1.2.10 (Zerbino and Birney, 2008) and GapCloser v1.12 (Zhao et al., 2011). Assembled sequences were confirmed by BWA v0.7.17 (Li, 2013) and SAMtools v1.9 (Li et al., 2009). All bioinformatic analyses were conducted in the Genome Information System (http://geis.infoboss.co.kr/) utilized in the previous studies (Kim et al., 2018, 2019a, 2019b, 2019c, 2021; Bum et al., 2020; Park et al., 2021c).

Genome annotation was conducted based on another *L. tetragonum* chloroplast (MW085088.1) with Geneious R11 v11.0.5 (Biomatters Ltd, Auckland, New Zealand). A circular map of *L. tetragonum* chloroplast genome was drawn using OGDRAW v1.31 (Greiner et al., 2019).

*Author for correspondence: starflr@infoboss.co.kr

Identification of intraspecific variations

Single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) were identified using the 'Find variations/ SNPs' function implemented in the Geneious R11 v11.0.5 (Biomatters Ltd, Auckland, New Zealand) based on the pair-wise alignment of the two chloroplast genomes of *L. tetragonum* conducted by MAFFT 7.450 (Katoh and Standley, 2013). This method has been used in the previous studies (Kim et al., 2019a; Min et al., 2019a; Choi et al., 2021; Park et al., 2021b, 2021d). INDEL region was defined as the continuous INDELs.

Phylogenetic analysis

Maximum-likelihood (ML) and Bayesian inference (BI) phylogenetic trees were constructed based on the multiple sequence alignment of all available nine Plumbaginaceae chloroplast genomes and the seven chloroplast genomes of non-core Caryophyllales clade (Crawly and Hilu, 2012; Yao et al., 2019) by MAFFT v7.450 (Katoh and Standley, 2013). The chloroplast genome of *Nepenthes graciliflora* Elmer (1912) (Yao et al., 2019) was marked as outgroup species. The ML tree was reconstructed in IQ-TREE v1.6.12 (Nguyen et al., 2015) with 1,000 bootstrap repeats. In the ML analysis, a heuristic search was used with nearest-neighbor interchange branch swapping, GTR+F + R4 model determined as the best fit model by the ModelFinder implemented in IQ-TREE, and uniform rates among sites. All other options used the default settings. The posterior probability of each node was estimated by the BI using MrBayes v3.2.6 (Huelsenbeck and Ronquist 2001). The HKY85 model with gamma rates was used as a molecular model. A Markov chain Monte Carlo algorithm was employed for 1,100,000 generations, sampling trees every 200 generations, with four chains running simultaneously. Trees from the first 100,000 generations were discarded as burn-in.

Data availablity

Chloroplast genome sequence can be accessed via accession number of MN044572 in GenBank of NCBI at https://www.ncbi.nlm.nih.gov. The associated BioProject, SRA, and Bio-Sample numbers are PRJNA737051, SAMN19678692, and SRR14793527, respectively.

Results and Discussion

The chloroplast genome of *L. tetragonum* (GenBank accession of MN044572) isolated in Korea is 154,689 bp long (GC ratio of 37.0%) and had four subregions: 84,572 bp of large single copy (35.3%) and 12,813 bp of small single copy (31.5%) regions are separated by 28,562 bp of inverted repeat (IR; 40.9%) (Table 1), which is similar to those of the previously reported *L. tetragonum* chloroplast genome (MW085088) (Table 1). It contained 128 genes (83 protein-coding genes [PCGs], eight rRNAs, and 37 tRNAs); 14 genes (five PCGs, four rRNAs, and five tRNAs) were duplicated in the IR regions, which is also same to those of the previously reported *L. tetragonum* chloroplast genome (MW085088) (Fig. 1, Table 1).

Based on the pair-wise sequence alignment with the previously reported *L. tetragonum* chloroplast genome (MW085088) (Darshetkar et al., 2021), 35 SNPs and 33 INDELs regions (88 bp in total) were identified. The longest INDEL region was 18-bp located between *trnT* and *trnL*. The inserted sequence was 18-bp repetitive sequences. In addition, two chloroplast genomes of *Limonium bicolor* (Bunge) Kuntze (Darshetkar et al., 2021) display extremely divergent manner, no SNP and 116 INDEL regions (15,840 bp in length), which is similar to the case of *Phedimus takesimensis* chloroplast genomes (129 SNPs and 112 INDEL regions (8,506 bp in length) (Park et al., in preparation).

Numbers of these intraspecific variations of *L. tetragonum* were smaller than those of *Pseudostellaria palibiniana* (Takeda) Ohwi (84 SNPs and 125-bp INDELs), *Pyrus ussuriensis* Maxim. (1,221 SNPs and 781-bp INDELs), *Goodyera schlechtendaliana* Rchb. f. (200 SNPs and 511-bp INDELs), *Gastrodia elata* Blume (324 SNPs and 630-bp INDELs) isolated in Korea; while they were larger than those of *Artemisia fukudo* Makino (seven SNPs and 12-bp INDELs) (Min et al., 2019b), *Fagus multinervis* Nakai (two SNPs and 2-bp INDELs) (Park and Oh, 2020), *Aconitum coreanum* (H. Lév.) Rapaics (five to 19 SNPs and 52-bp to 950bp INDELs) (Kim et al., 2019d), *Viburnum erosum* Thunb. (16 SNPs and 50-bp INDELs) (Choi et al., 2020), and *Veronica nakaiana* Ohwi (seven SNPs and 4-bp INDELs) (Lee et al., 2021). In

Table 1. List of two available chloroplast genomes of *Limonium tetragonum.*

GenBank accession	Length (bp)				GC contents				No. of genes		
	Whole	LSC	SSC	IR	Whole (%)	LSC (%)	SSC (%)	IR (%)	No. of PCGs	No. of tRNAs	No. of rRNAs
MN044572	154,689	84,572	13,013	28,562	37.0	35.3	31.5	40.9	83	37	8
MW085088	154,691	84,568	12,997	28,563	37.0	35.3	31.5	40.9	83	37	8

LSC, large single copy; SSC, small single copy; IR, inverted repeat ; PCG, protein-coding gene.

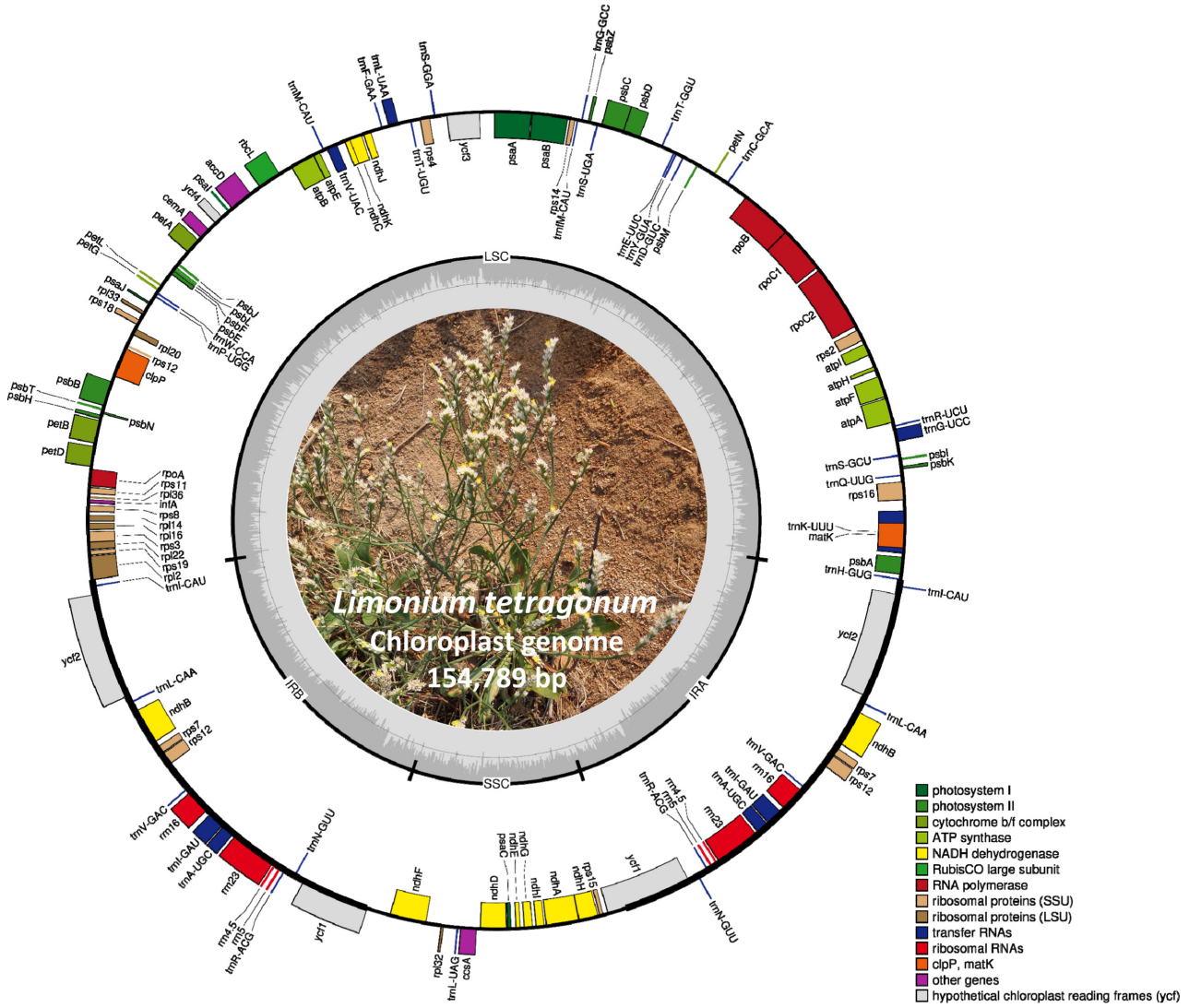

Fig. 1. Circular map of chloroplast genome of *Limonium tetragonum* isolated in Korea. Genes shown outside are transcribed clockwise, and those inside the circle are transcribed counter clockwise. Genes are color-coded to distinguish different functional groups. The dark grey and the light grey plot in the inner circle correspond to the GC content and AT content, respectively.

addition, seven PCGs contained at least one SNP or INDELs, among which two synonymous SNPs were found in *psbA* and *psaA* (Table 2) and four non-synonymous SNPs were identified in *rpl20*, *rpl32*, and *ycf2* located in the IR region (Table 2). The ratio of synonymous to non-synonymous SNPs was 0.5, which is different from the normal ratio, like *Chenopodium album* L. (Park et al., 2021a). One INDEL region was identified in the genic region of *rpoC2*, resulting the extension of *rpoC2* length in comparison to the previously sequenced chloroplast genome (MW085088) (Table 2).

Sixteen chloroplast genomes were used for constructing ML and BI phylogenetic trees. Both phylogenetic trees displayed that two *L. tetragonum*, collected from the East and West coastal areas of Korean peninsula, respectively, were clustered with high bootstrap value (Fig. 2). The sister species of *L. tetragonum* is controversial: *L. tetragonum* formed a sister group with *Limonium aureum* (L.) Mill. in both trees (Fig. 2), whereas it was clustered with *L. bicolor* in the previous study using chloroplast genomes (Darshetkar et al., 2021). The close relative of *L. tetragonum* was not clearly confirmed because the phylogenetic study using chloroplast and nuclear loci could not determine the relationship with *Limonium sinense* (Girard) Kuntze, *Limonium tenellum* (Turczaninow) Kuntze, and *Limonium flexuosum* (Linnaeus) Kuntze (Koutroumpa et al., 2018) and the study with the complete chloroplast genome of *L. tetragonum* displayed that *L. bicolor* was sister species

Table 2. List of intraspecific variations identified from the two *Limonium tetragonum* chloroplast genomes.

No.	Type	Changed bases		Coordination		Type	Position	SNP type
		MN044572	MW085088	MN044572	MW085088			
1	SNP	G	A	485	485	Genic	psbA	Synonymous SNP
2	INDEL	-	AA	4316	4316-4317	Intergenic	trnK-rps16	
3	SNP	G	A	4,549	4551	Intergenic	trnK-rps16	
4	INDEL	T	-	6,256	6258	Intergenic	rps16-trnQ	
5	INDEL	-	AA	6,670	6671-6672	Intergenic	trnQ-psbK	
6	INDEL	-	AAA	7,214	7217-7219	Intergenic	psbK-psbI	
7	INDEL	-	T	7,866	7872	Intergenic	trnS-trnG	
8	INDEL	-	CGGGTCA	7,966	7973-7979	Intergenic	trnS-trnG	
9	INDEL	-	A	8,224	8238	Intergenic	trnS-trnG	
10	INDEL	T	-	15,561	15576	Intergenic	rps2-rpoC2	
11	INDEL	-	T	15,898	15912	Genic	rpoC2	Frameshift
12	SNP	T	G	27,306	27321	Intergenic	rpoB-trnC	
13	SNP	T	G	27,307	27322	Intergenic	rpoB-trnC	
14	SNP	C	G	27,308	27323	Intergenic	rpoB-trnC	
15	SNP	C	A	27,309	27324	Intergenic	rpoB-trnC	
16	SNP	C	A	27,310	27325	Intergenic	rpoB-trnC	
17	INDEL	-	A	28,271	28286	Intergenic	trnC-petN	
18	INDEL	A	-	31,349	31435	Intergenic	trnE-trnT	
19	INDEL	-	AA	31,472	31487-31488	Intergenic	trnE-trnT	
20	SNP	C	A	31,768	31785	Intergenic	trnE-trnT	
21	SNP	A	G	32,417	32434	Intergenic	trnT-psbD	
22	INDEL	AAAG	-	36846-36849	36863	Intergenic	psbZ-trnG	
23	SNP	G	A	42,109	42122	Genic	psaA	Synonymous SNP
24	SNP	A	T	42931	42,944	Intergenic	psaA-ycf3	
25	SNP	A	T	42932	42,945	Intergenic	psaA-ycf3	
26	INDEL	ATATATTTATAT ATTATA	-	47679-47696	47,692	Intergenic	trnT-trnL	
27	INDEL	-	TAAT	47873	47868-47871	Intergenic	trnT-trnL	
28	INDEL	ATTTAAA	-	48687-48693	48,686	Intronic	trnL	
29	SNP	A	G	49,304	49296	Intergenic	trnF-ndhJ	
30	INDEL	-	AAAA	55,296	55288-55291	Intergenic	atpB-rbcL	
31	INDEL	-	T	55,474	55,470	Intergenic	atpB-rbcL	
32	INDEL	-	A	57,897	57,894	Intergenic	rbcL-accD	
33	INDEL	A	-	61,499	61,497	Intergenic	ycf4-cemA	
34	INDEL	-	TCTA	63,882	63879-63882	Intergenic	petA-psbJ	
35	SNP	G	T	64,137	64138	Intergenic	petA-psbJ	
36	SNP	A	T	64,138	64139	Intergenic	petA-psbJ	
37	SNP	A	T	64,139	64140	Intergenic	petA-psbJ	
38	SNP	T	G	64,140	64141	Intergenic	petA-psbJ	

Table 2. Continued

No.	Type	Changed bases		Coordination		Type	Position	SNP type
		MN044572	MW085088	MN044572	MW085088			
39	SNP	G	T	64,141	64142	Intergenic	petA-psbJ	
40	SNP	A	T	64,142	64143	Intergenic	petA-psbJ	
41	SNP	G	T	64,143	64144	Intergenic	petA-psbJ	
42	SNP	A	C	64,146	64147	Intergenic	petA-psbJ	
43	SNP	A	T	64,147	64148	Intergenic	petA-psbJ	
44	SNP	A	C	64,148	64149	Intergenic	petA-psbJ	
45	SNP	C	A	64,149	64150	Intergenic	petA-psbJ	
46	SNP	A	T	64,150	64151	Intergenic	petA-psbJ	
47	SNP	A	T	64,151	64152	Intergenic	petA-psbJ	
48	SNP	A	C	64,152	64153	Intergenic	petA-psbJ	
49	INDEL	T	-	65,511	65512	Intergenic	psbE-petL	
50	INDEL	T	-	66,150	66,150	Intergenic	petL-petG	
51	INDEL	TA	-	68210-68211	68,209	Intergenic	rpl33-rps18	
52	INDEL	-	C	68,295	68,292	Intergenic	rpl33-rps18	
53	SNP	T	G	69,126	69124	Genic	rpl20	Non-synomynous SNP
54	INDEL	CTTT	-	69359-69362	69,357	Intergenic	rpl20-rps12	
55	SNP	C	A	71,121	71115	Intronic	clpP	
56	INDEL	A	-	75,382	75,376	Intergenic	psbH-petB	
57	INDEL	A	-	80,610	80,603	Intergenic	rps8-rpl14	
58	INDEL	-	AAAA	81,060	81052-81055	Intergenic	rpl14-rpl16	
59	SNP	C	A	86,943	86939	Genic	ycf2	Non-synomynous SNP
60	INDEL	-	G	92,158	92,154	Intergenic	ycf2-trnL	
61	SNP	A	T	106,466	106463	Intergenic	trnR-trnN	
62	SNP	A	T	115,749	115746	Intergenic	ndhF-rpl32	
63	SNP	G	A	116,280	116277	Genic	rpl32	Non-synomynous SNP
64	INDEL	-	T	116,808	116805	Intergenic	rpl32-trnL	
65	INDEL	-	TTT	123,377	123375-123377	Intronic	ndhA	
66	SNP	T	A	132,796	132797	Intergenic	trnN-trnR	
67	INDEL	-	C	147,096	147097	Intergenic	trnL-ycf2	
68	SNP	G	T	152,319	152321	Genic	ycf2	Non-synomynous SNP

SNP, single nucleotide polymorphism; INDEL, insertions and deletion.

(Darshetkar et al., 2021), however, number of taxa used in this study was limited due to lack of complete chloroplast genomes and *L. bicolor* was not included in the previous study. This incongruency of the phylogenetic relationship might be caused by different sequences in both studies. Moreover, the supportive value of the clade of *L. tetragonum* and *L. bicolor* was 73%, which was lower than those (100%) in this study (Fig. 2), suggesting that *L. aureum* might be a sister species of *L. tetragonum*. Most of *Limonium* phylogenetic studies were conducted with the lack of East Asian *Limonium* species (Lledó

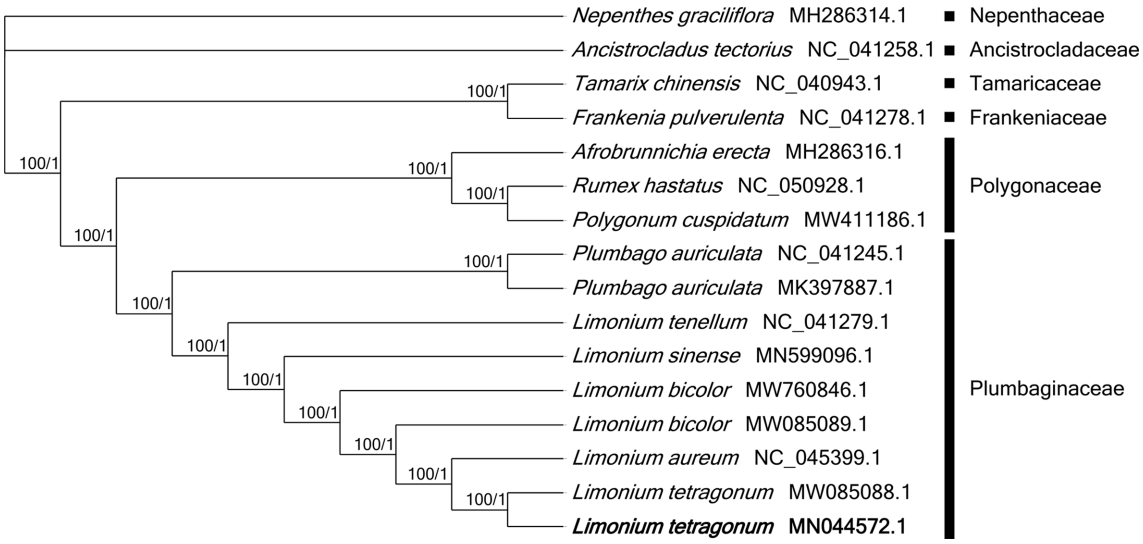

Fig. 2. Maximum-likelihood (ML) and Bayesian inference (BI) phylogenetic trees of 16 chloroplast genomes. Phylogenetic tree was drawn based on ML tree. The numbers above branches indicate support values of ML and BI trees, respectively.

et al., 2005; Malekmohammadi et al., 2017; Koutroumpa et al., 2018), requiring the additional phylogenetic studies including East Asian *Limonium* species.

ORCID: Yongsung KIM http://orcid.org/0000-0002-5349-9226; Jongsun PARK http://orcid.org/0000-0003-0786-4701

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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