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RESEARCH ARTICLE **Bhartiya Krishi Anusandhan Patrika**

Biochemical Analysis and DNA Barcoding of Millet *Echinochloa frumentacea*

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ABSTRACT

Background: Millets are small grains that are rich in nutrients. In recent times, millet-based foods have been increasingly recommended for a healthy diet. Many millets are not annotated or DNA barcoded yet.

Methods: In this study, comparative biochemical analyses especially that of starch and total protein of *Echinochloa frumentacea*, called as Indian barnyard white millet (Varai), from geographically different locations like Tamil Nadu and Maharashtra have been done. Their DNA barcoding has also been done to identify them on the basis of molecular data.

Result: It was observed that starch granules were more abundant in Tamil Nadu variety as compared to Maharashtra variety. Blue value, indicative of amylose: amylopectin ratio was found to be low in Varai, indicating that Varai has low starch digestibility and its starch releases glucose slowly, thus making it a low glycaemic index food. Protein content was higher in Tamil Nadu variety, but overall Varai had a lower protein content as compared to other millets. Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene from plastid was isolated, amplified by PCR, sequenced and the sequence was submitted to GenBank, NCBI. The gene was identified to be that of *Echinochloa frumentacea* and was given the accession numbers by GenBank as OR027010 (Varai, Maharashtra) and OR027011 (Varai, Tamil Nadu). This study indicated a distinct biochemical difference related to the geographical location of millets. This study helped barcoding of *Echinochloa frumentacea* Indian varieties using rbcL gene. This will further help in studies of phylogeny and evolution and also that of the relatedness of *Echinochloa sp* within and as compared to other millets.

Key words: Biochemical analysis, DNA barcoding, *Echinochloa frumentacea,* Millets, rbcL gene, Varai.

INTRODUCTION

Millets are small grains that have a rich nutrient profile. Nowadays, millet-based foods have been increasingly recommended for a healthy diet and to solve many health issues (Anitha *et al*., 2022). Millet consumption could thus help achieve SDG 3 (Sustainable Development Goal 3-Good health and well-being). Modern food systems may not offer essential nutrients. It is suggested that change in the food habits, including the promotion of local foods like millets, is essential (Pradhan *et al*., 2021). *Echinochloa frumentacea*, commonly called as Varai, or Indian barnyard millet, belongs to the family Poaceae and subfamily *Panicoideae*. It includes 250 annual and perennial species, of which *E. frumentacea* (Indian barnyard millet) and *E. esculenta* (Japanese barnyard millet) are the most important and widely cultivated (Farooq and Siddique, 2023). Varai is grown on fertile, free-draining, sandy loam soils. It is mainly grown in Kharif season, grown best in tropical conditions.

Panozzo *et al*. (2021) have DNA barcoded 2 species of *Echinochloa* namely *E. crus-galli* and two in *E. oryzicola* but not *frumentacaea.* Hoste *et al*. (2022) have given a key for correct identification of *Echinochloa species* including *frumentacea* and have emphasized the need of efforts in morphology-based taxonomy, genomics and phylogenetics to overcome the confusion among the *Echinochloa sp* which are major weeds in rice and maize fields. Ceaser and Maharajan (2022) have stated that genome sequences of many millets have not been annotated, which may hamper millet research, which is very important for food security and attaining UN SDGs. Gao *et al*. (2022) have done a comparative analysis of whole chloroplast genomes of *Echinochloa sp.* Omonhinmin and Onuselogu (2022), in their article have emphasized the importance of Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit rbcL gene as global molecular data repositor, which can help in finding relatedness of plants and study evolutionary relationships.

The aim of this study was to select and collect millets from various regions of India and perform biochemical analyses on them, including microscopic analysis of starch granules in millets, extraction and estimation of total proteins and estimation of the amylose: amylopectin ratio via Blue Value determination. The other aim of this study was to sequence plastid rbcLgene and to barcode *Echinochloa frumentacea* Indian varieties from Maharashtra and Tamil Nadu.

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MATERIALS AND METHODS

This project was conducted during the period from June 2022 to Augsut 2023, at the Department of Life Science, Ramnarain Ruia Autonomos College, Mumbai. The genomic studies were conducted in collaboration with GeneOmbio Technologies Pvt Ltd, Pune.

Selection and collection of millets from different regions in India

Millet *Echinochloa frumentacea* (Varai) was obtained from local markets in Mumbai (Maharashtra) and Chennai (Tamil Nadu). Varai is generally grown on fertile, free-draining , sandy loam soils. It is mainly grown in *Kharif* season. It is a short duration crop, grown best in tropical conditions.

Biochemical analysis

Microscopic examination of millet starch granules

The samples were soaked overnight in water and then sectioned, stained by dilute iodine and observed under the microscope (45×) for analysis of starch granule shape and abundance.

Extraction and estimation of total proteins

The millets were powdered. 1 g of each sample was weighed, 4 ml of hexane was added and the mixture was kept for 4 hours. This was the defatting step. Then they were centrifuged at 4000 rpm for 40 min, the supernatant was removed and in the pellet, 8 ml distilled water was added and kept for 4 hours. Again, the samples were centrifuged and the supernatants were estimated for their protein content by Lowry's method (Plummer, 2013).

Estimation of the amylose: amylopectin ratio by determination of the Blue Value (Nwokocha, 2014)

Millets were soaked overnight and then ground in mortar and pestle using distilled water, later filtered through muslin cloth. After the starch settled, the filtrate was decanted and then the starch was dried. 0.1 g dry starch sample was weighed in a tube, 1 ml ethanol (95%) was added followed by 9 ml of 1 M NaOH solution and heated in a boiling water bath for 40 min to solubilize the starch. The starch solution was cooled and transferred into a 100 ml standard volumetric flask and the volume was made up to the 100 ml mark with distilled water. 2.5 ml of starch solution was taken into a 50 ml standard flask; 0.5 ml of 1 M acetic acid was added, followed by 1 ml of stock iodine (0.2 g I2 and 2.0 g KI per 100 ml) and the solution was made up to the 50 ml mark with distilled water. The color was allowed to develop for 20 minutes and then the absorbance reading was measured at 620 nm using a UV/visible spectrophotometer. In the reference cell, an iodine solution of the same concentration as above but without starch was used. The blue value was calculated according to the method of Gilbert and Spragg (1964) using the formula:

$$\text{Blue value} = \frac{\text{Absorbance at 620 nm} \times 4}{\text{Concentration (mg/dl)}}$$

DNA barcoding using rbCLa gene

Genomic DNA isolation

DNA was isolated using Macherey Nagel Nucleospin kit, as per manufacturer's instructions.

PCR of rbcL gene

Plant rbcL region gene was amplified using standard PCR reaction. The primer pair rbcL-F and rbcL-R (Table 1) was used in PCR reaction with an annealing temperature of 57°C. After amplification, products were purified by using exosap kit (Invitrogen) and were directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at BlastN site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). DNA sequencing was performed using one of the PCR primers. The PCR reaction was performed in Applied Biosystems 2720 thermal cycler. Thermal cycling program for PCR used was; initial denaturation at 95°C for 5:00 min, denaturation at 94°C for 0:30 sec, annealing at 57°C for 0:30 sec, extension at 72°C for 0:30 sec, followed by final extension at 72°C for 10 min. This was repeated for 35 cycles and final hold at 4°C until use.

Following PCR mix was prepared for all DNA samples along with a negative PCR control and a positive control (Certified Reference Material). Final volume of each reaction was 25.0 µl. The reaction mix was prepared for all samples and added into 200 ìl PCR tubes. Genomic DNA was added later to each tube. PCR reaction mixture used was Genomic DNA 5 µl, 10X PCR buffer 2.5 µl, 50 mM $MgCl_2$ 0.75 µl, 0.5 mM dNTP Mix 0.5 µl, 10 pmole primer solution 1.00 µl, DMSO 1.25 µl, Taq DNA polymerase (5.0 units/ µl) 0.2 µl and Nuclease free water 13.8 µl.

Agarose gel (2%w/v) spiked with nuclear stain dye Labsafe (1:65000 diluted) was prepared Agarose (LE, Analytical Grade, Promega Corp., Madison, WI 53711 USA) in 0.5× TBE buffer. 5.0 ml of PCR product was mixed with 1 ml of 6× Gel tracking dye. 5 ml of gScale 100 bp+3k DNA Ladder (ExcelBand, SMOBIO) was loaded in one lane for confirmation of size of the amplicon using reference ladder. The DNA molecules were resolved at 5V/cm until the tracking dye was 2/3 distance away from the lane within the gel. Bands were detected under a UV Trans illuminator. Gel images were recorded using BIO-RAD GelDoc-XR gel documentation system. The PCR product of size ~599 bp was expected to be generated through this reaction.

Purification of PCR products

To remove unused dNTPs and primers from the reaction mixture, 10 ml PCR product was used for ExoSAP purification. ExoSAP-ITTM PCR Product Cleanup Reagent (Thermo Fisher) was used for enzymatic cleanup of amplified PCR product. Excess primers and nucleotides were hydrolysed in a single step. Purified PCR samples were further used for DNA sequencing.

DNA sequencing

ExoSAP purified PCR products (50 ng) were used for DNA sequencing. ABI BigDye ® Terminator v3.1 Cycle Sequencing reaction kit (Applied Biosystems, USA), was used. Sequencing reaction composition for 10 µl sequencing used was; PCR product DNA (3.00 µl), Sequencing buffer (1.90 µl), RR-100 (Ready Reaction Mix) (0.25 µl), 10pmole sequencing primer (1.00 µl) and Nuclease free water (3.85 µl).

Sequencing reaction was run in 2720 Thermal Cycler (Thermo Fisher) in standard sequencing program: 25 cycles of (96°C for 10 sec, 50°C for 10 sec, 60°C for 4 min), then ramp to 4°C.

Cycle sequencing PCR products were then purified by EDTA-Ethanol precipitation protocol. The cleaned-up sequencing products were dried at 37°C for 30 minutes and then dissolved in HiDIFormamide solution (10 µL).

The reaction tubes were then subjected to denaturation at 95°C for 3 min and snap chilling at 4°C. These products were loaded on Applied Biosystems DNA sequencing machine for capillary electrophoresis. Machine: 3130 Genetic analyzer Automated DNA sequencing machine Softwares used: Sequencing Analysis 5.1; ChromasPro v3.1.

DNA sequence analysis

DNA sequences were generated in FASTA format in sequencing machine and further analyzed by Sequencing Analysis 5.1 software. Using forward and reverse strand sequences a contig of trimmed sequence was generated. For each sample one FASTA sequence was thus generated and further analyzed. BLAST analysis-Sequencing similarity of the samples sequence with Genbank Database sequences was analysed by nucleotide BLAST. Clustal W-Clustal W alignment was used for comparing different sequences and finding out similarity between them. MEGA 6-software was used for construction of phylogenetic tree for the sequences by including the nearest matching reference sequences from NCBI Genbank nucleotide sequence database (Saitou, 1987; Kumar, 2018).

RESULTS AND DISCUSSION

Biochemical analysis

Microscopic analysis of starch granules

There was a distinct geographical difference in the shape, size *and* abundance of starch granules in the samples collected from Maharashtra and Tamil Nadu (Fig 1).

Protein estimation

Total protein was calculated using the equation from the standard protein graph (Fig 2). Varai from Maharashtra showed lesser total protein content as compared to that from Tamil Nadu (Table 2). Overall Varai has a lower protein content as compared to other millets. (Gopalan, 2011).

Estimation of amylose: amylopectin ratio by determination of Blue Value

Blue Value indicates the amylose to amylopectin ratio, which in turn indicates starch digestibility. Varai illustrated very low Blue Values (Table 3), which indicated presence of higher amounts of amylopectin in its starch, less digestibility of starch, a slower glucose release *and* thus a low glycaemic index. Regional variation in Blue Values is observed. Villas *et al.* (2019), have concluded in their study that high amylose content makes digestion easier while high amylopectin interferes in the digestion, thus molecular structure has a strong influence on starch digestibility.

Agarose gel electrophoresis of plant genomic DNA

Genomic DNA was extracted and detected by Agarose Gel Electrophoresis (Fig 3).

Polymerase Chain reaction

Samples showed PCR amplicon of desired size of ~599 bp on agarose gel (Fig 4).

DNA sequencing

Source: Chloroplast *Echinochloa frumentacea.*
Organism: *Echinochloa frumentacea*.

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliopsida; Liliopsida; Poales; Poaceae; PACMAD clade; Panicoideae; Panicodae; Paniceae; Boivinellinae. *Echinochloa*.

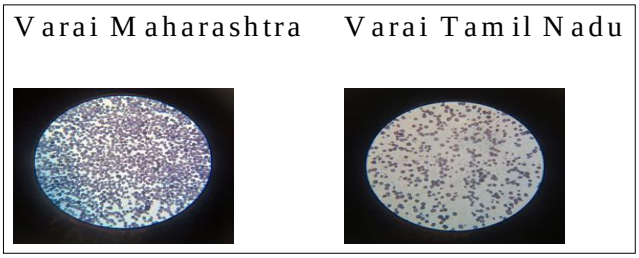


Fig 1: Comparative microscopic analysis of starch granules of Varai from Maharashtra and Tamil Nadu (45×).

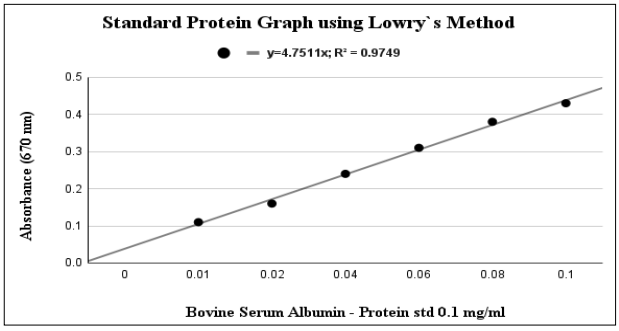


Fig 2: Std protein graph by Lowry's method.

Table 1: Forward and Reverse primers for rbcL PCR.

Name	Sequence 5′-3′	Bases	Amplicon size
rbcL-F	ATGATAACTCGACGGATCGC	20 bases	~599 bp
rbcL-R	CTTGGATGTGGTAGCCGTTT	20 bases	~599 bp

Table 2: Protein content of Varai (mg/ml per gram of millet).

Sample	Protein sample (ml)	Distilled water (ml)	OD at 670 nm	Calculated protein content based on std equation y= 4.7511×	Dilution factor 10 mg/ml
Varai T	0.1	0.9	0.23	0.048	0.48
Varai M	0.1	0.9	0.07	0.015	0.15

Table 3: Blue Values of Varai.

Sample	Absorbance at 620 nm	Calculated Blue Value (Abs × 4) /100=Abs × 0.04 mg/dl
Varai M	0.07	0.0028
Varai T	0.09	0.0036

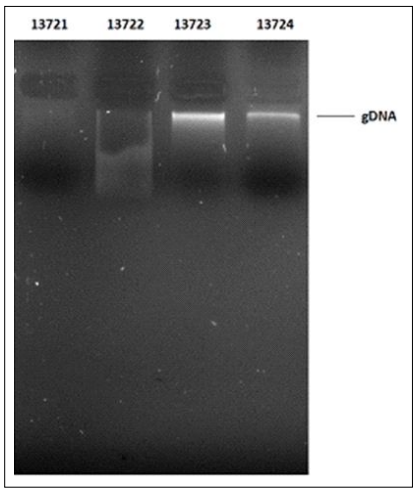
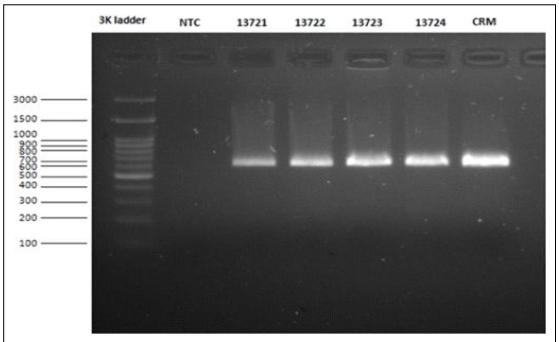


Fig 3: Agarose gel electrophoresis of genomic DNA performed on 1% (w/v) gel.



Lanes 3-6: 13721-13724 sample PCR Product. Lane 7: CRM (Certified Reference Material) or Standard positive control for plant.

Fig 4: PCR Image: 2% (W/V) Agarose gel electrophoresis: Lane 1: 100-1000+3k DNA marker; Lane 2: NTC (Negative test control).

Following sequences were generated for the sample

>13721 (rbcL) Varai M (Assembled Contig). Sequence was submitted to GenBank. Accession number given by GenBank OR027010;

Base count- 154 a 113 c 133 g 163 t

Origin

1 actaaagcaa gtgttggatt taaagctggt gttaaggatt ataaattgac ttactacact
61 ccggagtacg aaaccaagga tactgatatc ttggcagcat tccgagtaac tcctcagccc
121 ggggttccgc ctgaagaagc aggggctgca gtagctgcgg aatcttctac tggtacatgg
181 acaactgttt ggactgatgg acttaccagt cttgatcgtt acaaaggacg atgctatcac
241 atcgagcccg ttcctgggga ggcagatcaa tatatctgtt atgtagctta tccattagac
301 ctatttgaag agggttctgt tactaacatg tttacttcca ttgtgggtaa cgtatttggt
361 ttcaaagccc tacgcgctct acgtttggag gatctacgaa ttcccattgc ttatgcaaaa
421 actttccaag gtccgcctca cggtatccaa gttgaaaggg ataagttgaa caagtatggt
481 cgtcctttat tgggatgtac tattaaacca aaattgggat tatccgcaaa aaattacggt
541 agagcgtgtt atgagtgtct acg
/translation="TKASVGF KAGVKDYKLTYYTPEY ETKDTDILAAFRVTPQ PGVPPEEAGAAVA AESSTGT WT TVWTDGLTSLDRY KGRCYHIEP VPGEADQYI CY VAY PL DLFEEGSVTNMFTSIVGNVFGFKALRALRLEDLRIPIAYAK TFQGPPHG IQVER DKL NKYGRPLLGCTI KPKLGLSAK NYGRACYECLR"

>13722 Varai T (Assembled Contig). Sequence was submitted to GenBank. Accession number given by GenBank OR027011.

Base count 148 a 111 c 130 g 159 t

Origin

1 ggatttaaag ctggtgttaa ggattataaa ttgacttact acactccgga gtacgaaacc
61 aaggatactg atatcttggc agcattccga gtaactcctc agcccggggt tccgcctgaa
121 gaagcagggg ctgcagtagc tgcggaatct tctactggta catggacaac tgtttggact
181 gatggactta ccagtcttga tcgttacaaa ggacgatgct atcacatcga gcccgttcct
241 ggggaggcag atcaatatat ctgttatgta gcttatccat tagacctatt tgaagagggt
301 tctgttacta acatgtttac ttccattgtg ggtaacgtat ttggtttcaa agccctacgc
361 gctctacgtt tggaggatct acgaattccc attgcttatg caaaaacttt ccaaggtccg
421 cctcacggta tccaagttga aagggataag ttgaacaagt atggtcgtcc tttattggga
481 tgtactatta aaccaaaatt gggattatcc gcaaaaaatt acggtagagc gtgttatgag
541 tgtctacg

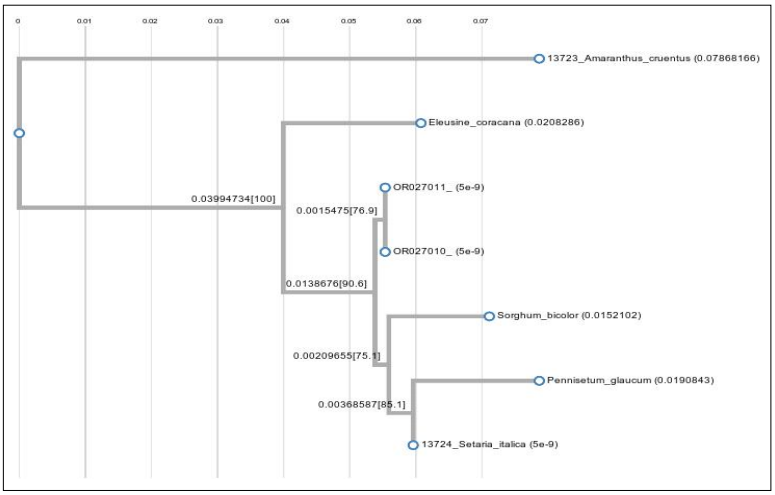


Fig 5: Relatedness of Varai with other millets.

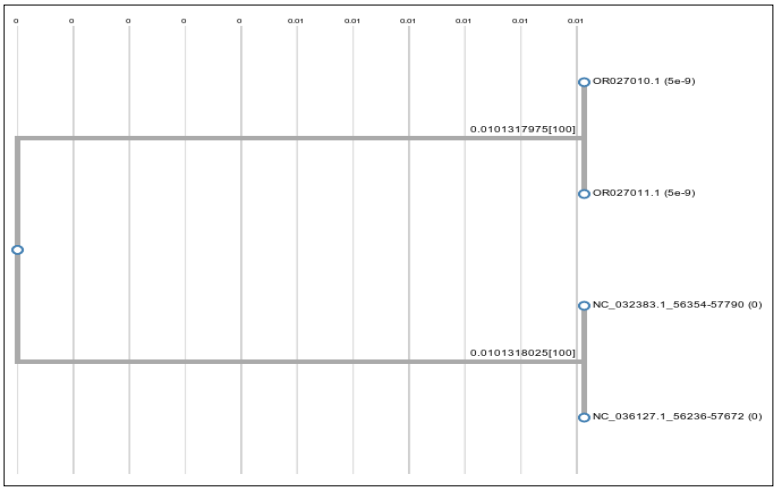


Fig 6: Phylogenetic tree of *E. frumentacea* (OR027010 and OR027011), *E. colona* (NC_ 032383) and *E. ugandensis* (NC_036127).

/translation="GFKAGVKDYKLTYYTPEYETKDTDILAAF RVTPQPGVPPEEAGAAVAAESSTGTWTTVWTDGLTSLDRY KGRCY HIEPVPG EADQYICY VAYPLDLFE EG SVTNMF TSIVGNVFGFKALRALRLEDLRIPIAYAKTFQGPPHGIQVERDKLN KYGRPLLGCTIKPKLGLSAKNYGRACYECLR"

In this study, the obtained sequences of Varai varieties were compared with sequences of other millets from NCBI database and phylogenetic tree was obtained (Fig 5). It illustrated that all millets have evolved together, whereas *Amaranthus* (Rajgira) has diverged from the millets, *Amaranthus* being correctly called as pseudo millet. Both the varieties of Varai were 100% identical when compared with each other. Varai showed distinct evolutionary deviation from *Eleusine coracana* (Ragi) (Fig 5).

Gao *et al*. (2022), based on the results of genetic relationships, divided 10 species of barnyard grass into four groups. The first group comprised *E. oryzicola*, *E. crus-galli* var. *zelayensis*, *E. glabrescens* and *E. stagnina*; the second group included *E. crus-galli* var. *crus-galli* and *E. esculenta*; the third group contained *E. haploclada* alone; and the fourth group consisted of *E. ugandensis*, *E. colona* and *E. frumentacea*. Fig 6 confirms the relatedness of these varieties.

DNA sequencing can help in improving agronomic traits, value addition in food, feed and nutritional security through recombinant technology. It can help in gene manipulation to create drought resistant crops. Thus it can lead the way to sustainable agriculture pertinent to the United Nations Accord of Sustainable Development Goals.

CONCLUSION

This study indicated a distinct biochemical difference related to the geographical location of millets. It has contributed to DNA barcode *Echinochloa frumentacea* Indian varieties using rbcL gene. The findings in this study will further help in studies of phylogeny and evolution and also that of the relatedness of *Echinochloa sp* within and as compared to other millets. It can be helpful in generating biotechnological interventions in food security and generating drought resistant crops. Besides, this study emphasized the importance of millets, 2023, being declared as International Year of Millets.

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Ethics statement

NA.

Conflict of interest

The authors declare that they do not have any conflicts of interest.

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