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Assessing DNA barcodes species discriminating ability and phylogenetic relation within *Embelia* species

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ABSTRACT

Member of *Embelia* is economically important medicinal plants. *Embelia ribes* is red listed endangered species. DNA barcodes Interspersed Transcribed Spacer (ITS), *matK*, *rbcL,* and *psbA-trnH* were selected for its species discrimination ability using TaxonDNA software and tree-based maximum likelihood monophyly analysis. The results showed that the species identification success rate of *psbA-trnL* and ITS+ *psbA-trnL* combination has high success rate "best match" and "best close match" functions on TaxonDNA. Among the barcodes employed, plastid *psbA-trnL* showed the highest success rate followed by ITS and *rbcL* while *matK* had the weak discriminating ability. In the concatenated sequences, the high discriminating rate was seen in ITS+*psbA*, ITS+*matK*+*rbcL*, and ITS+*matK*+*rbcL*+*psbA*. In the tree-based analysis, disjunct distribution of the *E. ribes* species was observed in the ITS phylogeny. It is necessary to know the discriminatory ability of single or combinations of barcodes to detect variation at the inter and intraspecies levels.

1. INTRODUCTION

In tropical and subtropical climatic areas across the world, there are numerous species of *Embelia*. *Embelia* is climber/shrubs belonging to the sub-family Myrsinoideae of family Primulaceae. *Embelia ribes* is commonly known as Vidanga which is a Red-listed species (Low Risk-Near Threatened) [1]. In the traditional medicine systems Ayurveda, Siddha, and Unani parts of *E. ribes* are utilized. It is hard to distinguish the fruit of *E. ribes* from black pepper therefore pepper is added as an adulterant. Members of the Myrsinoideae family are economically, medicinally important plants. Seed and fruits of the *Embelia* species contain marker molecule embelin [2].

E. ribes is in high demand due to its use of Ayurvedic formulations, but it is frequently misidentified as *Embelia basaal* [1] and *Embelia robusta* [3]. Due to its growing demand *E. ribes*, is a commercially grown [4,5]. However, due to over-exploitation, depleting forest area, seed dormancy, embryos abortion, and hard seed coat, regeneration of the crop is poor [6]. There is the gradual disappearance of *E. ribes* due to the inherent sterility of the seeds [7,8]. Comparison of floral ontogeny of two each of *Maesa and Embelia* species, and *Aegiceras corniculatum* has shown phyllotaxy of 2/5-spiral development with few minor variations [9].

Phylogenetic analysis based on morphological characters, nuclear and chloroplast barcoding genes have been studied exhaustively for the genera belonging to family Primulaceae. Devaiah and Venkatasubramanian [10] have designed sequence characterized amplified region and also Random amplified polymorphic DNA (RAPD) marker for *E. ribes* to distinguish from the adulterants [11]. Amplified fragment length polymorphism has been used to detect polymorphisms in *E. ribes* and *Embelia tsjeriam-cottam* [12]. Chrungoo *et al*. have used Inter Simple Sequence Repeat (ISSR) primer and DNA barcode regions Interspersed Transcribed Spacer (ITS) and *matK* to check genetic diversity of *E. ribes*, *Embelia subcoraceae*, *Embelia floribunda* sampled from the North-east region of India. In a study by Bajpe *et al*. (2018), using DNA markers RAPD and ISSR the genetic variation within and between *Embelia* species, *Maesa indica*, and *Ardisia solanacea* from Karnataka's the Western Ghats was analyzed.

To identify plants species a number of different DNA sequences regions been proposed; however, *matK*+*rbcL* is selected as core DNA barcodes along with ITS and *trnH–psbA* sequence [13]. Although these loci offer several advantages, it has long been recognized that no one locus is appropriate for all plant species. The objective of the current study is to assess the species discriminatory ability of DNA barcode and also, to understand the phylogenetic relation within the *Embelia* species.

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2. MATERIALS AND METHODS

2.1 DNA Barcode Polymerase Chain Reaction (PCR), Sequencing, Homology Search

ITS4 and ITS5 sequence primers were used for PCR amplification of ITS1-5.8s-ITS2 sequence primers [14]. Primers (3FKIM and 1RKIM) designed by Ki-Joong Kim were used to amplify *matK* region following protocol described by the authors. Chromous Biotech, Bangalore sequenced the amplified products. Eighteen ITS sequences and six *matK* sequences were generated. After the sequences generated were submitted to the National Center for Biotechnology Information (NCBI) database, individual samples were granted accession numbers. Table 1 lists the sequences that have been deposited.

2.2. Phylogenetic Analyses and Species Discrimination

Multiple Sequence alignment (MSA) was carried out using Clustal W software. Phylogenetic trees were constructed using Mega X [15].

Table 1: The ability of four DNA barcodes and their combinations to discriminate *Embelia species.*

DNA Marker sequence	Sequence used	Species detected	Consensus sequence length (bp)
ITS	98	11	870
matK	23	10	1212
rbcL	22	9	1350
psbA-trnL	20	8	638

Using "Find best DNA model" within the Model feature of the Mega X software, model with lowest Bayesian information criterion (BIC) was obtained. With 1000 bootstrap (BS) replications, we built all the maximum likelihood (ML) phylogenetic trees applying best-fit DNA substitution model. Using the software Sequence Matrix, various barcode combinations were concatenated [16]. The Kimura 2-model was used to test the individual level discrimination rates for each marker using the "best match" (BM) and "best close match" (BCM) functions on TaxonDNA [17].

3. RESULT

3.1. Species Discrimination

Plastid *psbA* had the highest success rate in species discrimination (BC and BCM-64.28%), followed by nuclear ITS (BC and BCM-60.49%) and *rbcL* (BC and BCM-50%) among the barcodes used. The *matK* had the least discriminatory power (BC and BCM-34.78%). The highest discrimination rate was obtained when barcodes were combined is 64.19% in ITS+*psbA*, 62.79% in ITS+*matK*+*rbcL,* and 62.92% in ITS+*matK*+*rbcL*+*psbA* were observed [Table 2]. Within the ITS and its combination, there was a noticeable variation between BC and BCM.

3.2. Phylogenetic Analysis

3.2.1. Inter-transcribed spacer

The phylogenetic tree was generated using 99 ITS sequences. 98 sequences were of Genus *Embelia* and a sequence of *M. indica.* In the

Table 2: "Best match" and "best close match" TaxonDNA functions for identification of Western Ghats *Embelia* species.

Barcode	BM in %			BCM in %				T
	C	A	I	C	A	I	No match	
ITS	49 (60.49%)	10 (12.34%)	22 (27.16%)	47 (58.02%)	10 (12.34%)	19 (23.45%)	5 (6.17%)	3
matK	8 (34.78%)	7 (30.43%)	8 (34.78%)	8 (34.78%)	6 (26.08%)	8 (34.78%)	1 (4.34%)	2.23
rbcL	11 (50.0%)	8 (36.36%)	3 (13.63%)	11 (50.0%)	8 (36.36%)	3 (13.63%)	0 (0.0%)	1.56
psbA+*trnL*	9 (64.28%)	0 (0.0%)	5 (35.71%	9 (64.28%)	0 (0.0%)	5 (35.71%	0 (0.0%)	3.41
ITS+*matK*	51 (59.3%)	13 (15.11%)	22 (25.58%)	49 (56.97%)	13 (15.11%)	20 (23.25%)	4 (4.65%)	3
ITS+*psbA*	52 (64.19%)	8 (9.87%)	21 (25.92%)	49 (60.49%)	8 (9.87%)	20 (24.69%)	4 (4.93%)	3
ITS+*rbcL*	52 (59.77%)	12 (13.79%)	23 (26.43%)	50 (57.47%)	12 (13.79%)	21 (24.13%)	4 (4.59%)	3
matK+*rbcL*	13 (52.0%)	3 (12.0%)	9 (36.0%)	13 (52.0%)	3 (12.0%)	9 (36.0%)	0 (0.0%)	3.41
psbA+*rbcL*	14 (60.86%)	2 (8.69%)	7 (30.43%)	14 (60.86%)	2 (8.69%)	7 (30.43%)	0 (0.0%)	3.41
psbA+*matK*	13 (52.0%)	3 (12.0%)	9 (36.0%)	13 (52.0%)	3 (12.0%)	9 (36.0%)	0 (0.0%)	3.41
ITS+*matK*+*rbcL*	54 (62.79%)	11 (12.79%)	21 (24.41%)	51 (59.3%)	11 (12.79%)	20 (23.25%)	4 (4.65%)	3
ITS+*matK*+*psbA-trnL*	53 (59.55%)	12 (13.48%)	24 (26.96%)	51 (57.3%)	12 (13.48%)	22 (24.71%)	4 (4.49%)	3
rbcL+*matK*+*psbA-trnL*	16 (53.33%)	2 (6.66%)	12 (40.0%)	16 (53.33%)	2 (6.66%)	12 (40.0%)	0 (0.0%)	2.26
ITS+*matK*+rbcL+*psbA-trnL*	56 (62.92%)	10 (11.23%)	23 (25.84%)	53 (59.55%)	10 (11.23%)	22 (24.71%)	3 (4.49%)	3

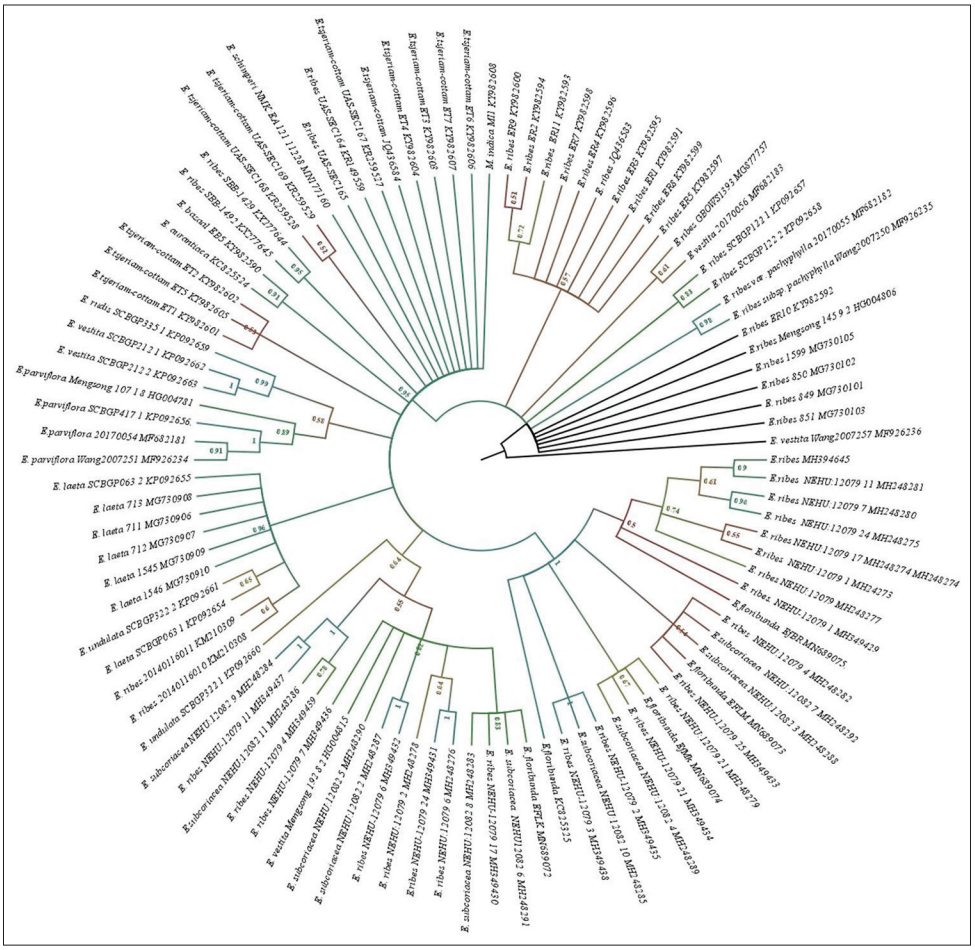

Figure 1: Phylogenetic tree of maximum likelihood bootstrap of the ITS sequences of *Embelia* species.

case of MSA, the overall sequence length was 1036 bp. In terms of BIC scores, the Kimura two-parameter with gamma distribution was the model with the lowest score (K2+G).

In the current cladogram, five clades can be observed within the genus *Embelia*. Within the phylogeny, discordant distribution of *E. ribes* was observed. *E. ribes* was nested along with other species samples, obtained from similar topographical regions. Clade 1 contains *E. ribes E. ribes var. pachyphylla* and *Embelia vestita* samples from the Yunnan region of China and Western Ghat samples with several polytomies were observed. Samples from the Western Ghats *E. basaal, Embelia aurantiaca,* and only sample from Africa *Embelia schimperi* was nested within the clade 2. Furthermore, in clade two, samples of *E. tsjeriam-cottam* from Western Ghats and *E. ribes* (two samples each from Southern and Northern India, respectively) were observed. Within Clade three, seven samples *Embelia laete* and two samples of *E. ribes* from China isolated identical topographical regions and samples of *Embelia undulata* grouped. Clade four was well resolved with *Embelia parviflora, E. vestita,* and with samples of *Embelia rudis* with good BS values. Clade five and six were well resolved paraphyletic clades with *E. ribes*, the samples of *E. floribunda*, *Embelia subcoriacea,* and *E. vestita* were nested along within the clade containing samples of Western Ghats of Karnataka and Northeast India and China [Figure 1].

3.2.2. matK sequence

MSA was performed on 23 *Embelia* species and outgroup *Maesa salicifolia* sequences. MSA yielded an overall sequence length of 931 bp, with 782 bp (87.86%) being conserved, 79 bp (9.08%) being variable sites, and 17 bp (1.92%) being parsimony informative sites. Tamura-Nei was the model with the lowest BIC scores (T92).

The samples of ER8, ER10, EB2, and EB3 of Western Ghats contained a 24 bp insert AAATATTTTGTAAAAGTATTTACT.

The cladogram of the *Embelia* species contained a major clade. The clade bifurcated to give two subclades very strong BS support values <30%. One of sub-clade contained *E. ribes* (from the Western Ghats and China), *E. basaal* and *E. tsjeriam-cottam* and another subclade contained *E. undulata, Embelia laeta*, *E. parviflora*, *E. vestita*, *E. rudis* from China, *Embelia xylocarpa* from Mozambique, and *Embelia australiana* and *E. parviflora* formed basal polytomy [Figure 2]. In phylogeny of *Embelia* species, the Western Ghats samples have formed a monophyletic group in both ITS and *matK* analysis. The discordant distribution is seen in samples from Northeast *Embelia* species, *E. subcoriacea, E. ribes*, and *E. ribes* from China.

3.2.3. rbcL

In the *rbcL* Cladogram, 27 sequences from the NCBI database were utilized with one outgroup. MSA resulted in an overall sequence length of 570 bp of which 518 bp (90.5%) were conserved, 79 bp (9.47%) were variable sites, and 15 bp (2.63%) were parsimony informative sites. The model with the lowest BIC scores was the Tamura-Nei model (T92).

A well-resolved two clades were observed within the phylogeny. Within Clade 1, three subclades were observed and polytomy of

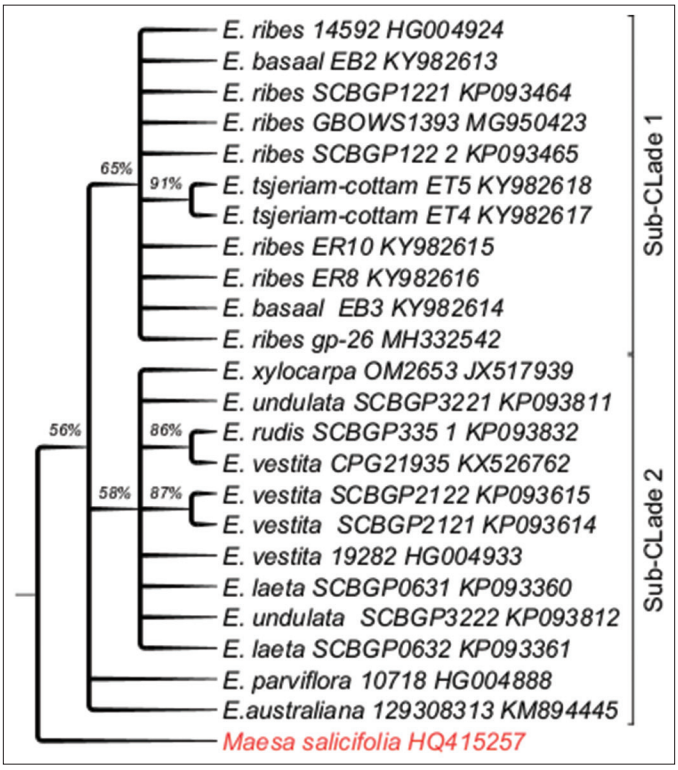

Figure 2: Phylogenetic tree of maximum likelihood bootstrap of the *matK* sequences of *Embelia* species.

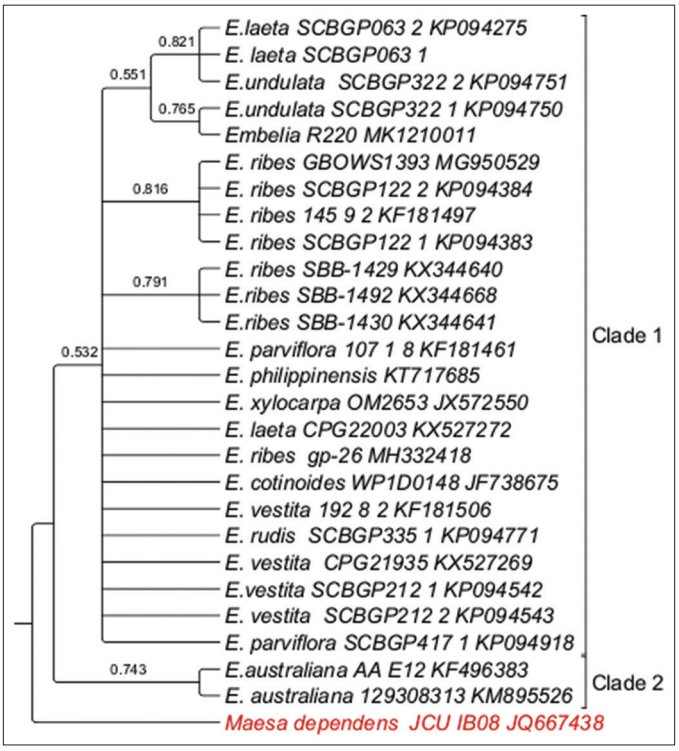

Figure 3: Phylogenetic tree of maximum likelihood bootstrap of the *rbcL* sequences of *Embelia* species.

several species from South East Asia such as China, the Philippines along with samples *Embelia xlocarpa* and *Embelia cotinoides* samples from Mozambique and Papua New Guinea, respectively. Two samples from Australia, *E. australiana* formed Clade 2. Node BS value of >50% was observed. The tip node BS of >than 75% was observed for *E. laeta, E. undulata, E. ribes,* and *E. australiana* [Figure 3].

3.2.4. psbA-trnL

In the *psbA-trnL* Cladogram, 27 sequences from the NCBI database were utilized with one outgroup. MSA resulted in an overall sequence length of 604 bp of which 177 bp (29.5%) were conserved, 423 bp (70.3%) were variable sites, and 165 bp (27.3%) were parsimony informative sites. The model with the lowest BIC scores was the Tamura-Nei model (T92).

A well-resolved clades were observed within the phylogeny. Within the Clade, four subclades were observed and polytomy of several species from South East Asia such as China, the Philippines along with samples *E. xlocarpa* and *E. cotinoides* samples from Mozambique and Papua New Guinea, respectively. Two samples from Australia, *E. australiana* formed Clade 2. Node BS value of > 50% was observed. The tip node BS of >than 75% was observed for *E. laeta, E. undulata, E. ribes,* and *E. australiana* [Figure 4].

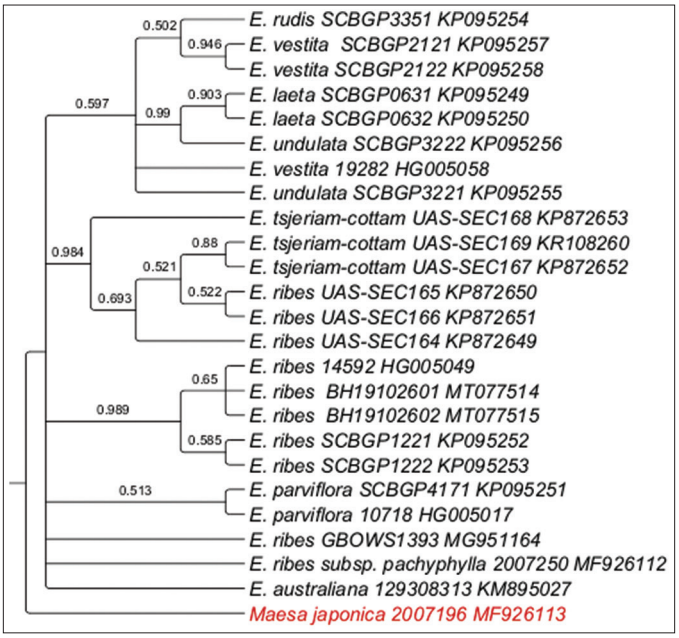

Figure 4: Phylogenetic tree of maximum likelihood bootstrap of the *psbA-trnL* sequences of *Embelia* species.

4. DISCUSSION

Barcodes for different plants have been studied extensively, but so far, no consensus has been reached. As a core barcode for plants, plastids region was initially proposed, but they have not been successful in all genus. ITS barcode was rejected by many researchers for inclusion in the core plant barcode region because it was challenging. Because of its lower intra-specific variation but larger inter-specific divergence, the ITS region has a high rate of proper classifying species. In several plant genera, combinations of ITS and plastid loci were discovered to be the optimum option [18]. Likewise for *Embelia* species, the ITS+*psbA-trnL* DNA barcode has similar discriminatory power as single barcodes. Similar results were observed in the species discrimination ability of *Salacia* species of Western Ghats [19,20]. Chen [21] proposed using ITS2 as a DNA barcode for fungus and green plants since it has a shorter sequence length than ITS and fast PCR amplification or sequencing rates [22].

Many researches have shown that *trnH–psbA* is a suitable plant DNA barcoding marker [23]. The high species discrimination ability of *psbA-trnL* could be attributed to a low number of sequences deposited in the NCBI database. ITS sequences in the NCBI database were found to be greater than the *matK*, *rbcL*, and *psbA-trnL* [18]. In the current study, we could observe that the *psbA-trnL* has more variable site than the ITS sequences. As a result, the *trnH–psbA* region in *Embelia* species is a promising barcode to be utilized as a single barcode. It was observed that in the NCBI database the sequences were mainly from the South East Asia especially from the India and China and only couples of representative's species such as *E. xylocarpa* from Mozambique, *E. australiana* from the Australia. Based on the TaxonDNA, the combination of *matK+rbcL* greatly improved the species discriminating rates to 52% from 34.78% and 50.0% respectively. At the species level, this combination's overall identification capability is poor.

The purpose of phylogenetic analysis is to determine the utility of a DNA region as a barcode for detecting species specific clusters from the same genus and family [24]. The discordant distribution of *E. ribes* was seen in all the phylogenetic tree. Polytomies were identified in the current study's barcode phylogeny, particularly in samples from the Western Ghats. This could be because *Embelia* species is a tropical species and mainly found is South East Asia, Western Ghats, and in Northern East parts of India.

TaxonDNA is used to evaluate various species, including *Lilium* species [25], *Aquilaria* species from China [26], and many more medicinal plants. TaxonDNA's BM and BMC indexes may occasionally be lower than those found in tree-based analyses. As a result, it is optimal to employ TaxonDNA in conjunction with Tree-based analysis to identify species. Small barcoding gaps, significant interspecies similarity, unknown origin, and ambiguous evolution all contributed to limited discrimination in TaxonDNA analysis [27].

5. CONCLUSION

It is essential to know the discriminatory power of single or combination of barcodes for proper identification and authentication of species, so that when new studies on *Embelia* species are undertaken only the DNA barcode/s with higher discriminatory power shall be chosen to detect variation at inter and intraspecies levels.

6. AUTHORS' CONTRIBUTIONS

All the authors have made substantive intellectual contributions to the content of this manuscript in the following areas: concept and design -SNB and KRK; data acquisition and analysis-SNB & KMM; drafting manuscript—SNB & RR; critical revision of manuscript SNB, ASB, KRM; and supervision- KRK, and final approval-SNB.

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8. FUNDING

Nil.

9. CONFLICT OF INTERESTS

We declare no conflict of interests regarding the publication of this manuscript.

10. ETHICAL APPROVALS

This article does not contain any studies conducted on human or animal subjects.

11. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

12. PUBLISHER'S NOTE

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