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RESEARCH ARTICLE

Length variation of chloroplast simple sequence repeats in the genus *Eucalyptus* L'Hér.

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ABSTRACT
Eucalyptus L'Hér. is an economically important genus of plants with several environmental significances and great industrial advantages. To accelerate breeding and conservation studies, efforts on molecular breeding and molecular genetic analysis are underway in the genus *Eucalyptus*. Despite these efforts, no sufficient information is available about common, polymorphic and unique chloroplast simple sequence repeats (cpSSRs) in the genus *Eucalyptus*. . These repeats consist of 1-6 nucleotides and play important role in the development of molecular markers, genetic mapping and plant breeding. In the present study, a total of 920 cpSSRs were detected and length variation of cpSSRs analysed between each pair of species among 31 chloroplast genome sequences of the genus *Eucalyptus*. Additionally, cross species transferability of common and polymorphic cpSSRs were also observed. The common, unique and putative polymorphic cpSSRs analysed in this study can be used for species identification and genetic diversity studies of *Eucalyptus*.

Introduction

Eucalyptus is a diverse genus of plants and world's driving industrial plantation species with multipurpose utility. It allows a great variety of uses with profitable and sustainable applications (1). Most of the *Eucalyptus* plantations all over the world are intended for paper, pulp and veneer production (2). Additionally, *Eucalyptus* has greater forest productivity and several environmental and medicinal significance (3-5). Efforts on molecular breeding and molecular genetic analysis are underway in *Eucalyptus* to accelerate breeding and conservation. Various molecular markers including restriction fragment length polymorphism (RFLP; 6), random-amplified polymorphic DNA (RAPD; 7) and amplified fragment length polymorphism (AFLP; 8) have been used in *Eucalyptus* for a variety of purposes. Currently, simple sequence repeats (SSRs) are widely applied molecular markers in genetic studies (1, 9, 10).

SSRs also known as microsatellites are tandem repetitions of short motifs of 1-6 nucleotides (11). These repeats are found in coding and non-coding regions of prokaryotic and eukaryotic genomes (12, 13). SSRs are broadly used genetic markers due to its codominant and highly reproducible nature. Along with phylogenetic studies, organelle genomes have also been used to identify SSRs (14-20).

Earlier, it was reported that 4 highly polymorphic genomic SSRs were characterized in *E. nitens* and observed 50% conservation of SSR loci in subgenera *Symphyomyrtus* and *Monocalyptus* (9). Complete chloroplast genome sequence of *E. globulus* was used to develop 35 cpSSRs, in which 10 cpSSR showed intraspecific polymorphism (21). Genetic diversity among 19 geographically defined *E. urophylla* populations was investigated using 12 nuclear SSR markers (22). Transferability was observed from their target species to closely related species (23, 24). In a study there developed 8 highly polymorphic genomic SSR loci for *E. leucoxylon* (25). EST-SSRs were also developed in many *Eucalyptus* spp (26 -28). There are reports on the identification of genetic markers in three *Eucalyptus* spp (*E. camaldulensis*, *E. tereticornis* and *E. grandis*) using high throughput method (29). Recently, distribution and organization of cpSSRs in 31 *Eucalyptus* species was observed (30).

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Apart from these efforts on SSRs have also been focused to identify putative polymorphic microsatellites from sequence data (13, 31, 32). These polymorphic SSRs have been differentiated from monomorphic SSRs by length variation between motifs. Recently, common and unique SSRs have also been identified in several species (13, 19, 20). Moreover, transferability of SSRs was observed between *Croton floribundus* and *Croton urucurana* (Euphorbiaceae;31) and genus *Lasiodiplodia* and *Neofusicoccum* (34).

Despite these efforts, no sufficient information is available about common, unique and putative polymorphic SSRs in chloroplast genomes (cp-genomes) of genus *Eucalyptus*. Therefore, the present bioinformatics study designed to analyse common, unique and putative polymorphic SSRs in complete chloroplast genome sequences of genus *Eucalyptus*.

Materials and Methods

Identification of common, unique and polymorphic cpSSRs

Complete chloroplast genome sequences of genus *Eucalyptus* were mined with the help of Microsatellite identification tool (MISA; http://pgrc.ipk-gatersleben.de/misa/misa.html) to detect common, unique and polymorphic cpSSRs. The minimum repeat size of ≥12 for mono-, ≥6 for di-, ≥4 for tri-, ≥3 for tetra-, penta- and hexa-nucleotide repeats was considered. The maximum interruption between two SSRs was taken as zero. A list of mined chloroplast genome sequences of genus *Eucalyptus* with their genome size and sequence accession number is given in Table 1.

Common, unique and putative polymorphic cpSSRs were detected using a recently developed methodology (13, 19). Briefly, identical repeating units with equal and varying length, showing significant similarity of flanking regions across the species, were considered as common and polymorphic SSRs respectively. Other repeating units and identical repeating units with no significant match of flanking regions across the species were considered as unique SSRs. A flowchart of the methodology used is shown in Fig. 1. Moreover, common and putative polymorphic cpSSRs with reference to chloroplast genome of *E. globulus* were illustrated using MapChart software (35).

Electronic PCR and gel simulation

The PCR primer pairs for selected common and polymorphic cpSSRs (with 200 nucleotides flanking region from both upstream and downstream regions of SSRs) were designed with default parameters (GC content minimum 30% and maximum 70% melting temperature minimum 57 and maximum 62 and PCR product size) using online Primer3 tool (http://bioinfo.ut.ee/primer3/; 36).

Electronic PCR with designed primer pairs were carried out to check PCR product and cross-species transferability of cpSSRs among the genus *Eucalyptus* using simulated gel electrophoresis tool SPCR (http://moleco.sjtu.edu.cn/spcr; 37). The threshold values 0.9 were set to upstream information coefficient (I_{up}), downstream information coefficient (I_{dn}), and product amplification coefficient (P_a).

Results and Discussion

Common, unique and polymorphic cpSSRs in genus Eucalyptus

In this study, 920 perfect cpSSRs were mined to analyse common, unique and polymorphic in *Eucalyptus* species. A total of 34 repeat units were detected which have distributed over the chloroplast genome sequence of genus *Eucalyptus*, in which 6 (19.35%) identical repeat units were found with varying length and 15 (48.38%) with same length. Additionally, 13 (41.93%) repeat units were observed as completely unique that were not found in the other cp-genomes. List of SSR motifs identified in cp-genomes of *Eucalyptus* species along with their frequency are presented in Table 2.

Mono-nucleotide motifs A/T, di-nucleotide motif AT, tri-nucleotide motif TTA, and tetra-nucleotide motifs ATAA/TTTC showed length polymorphism among the chloroplast cp-genomes of genus *Eucalyptus*. Additionally, 41 unique cpSSRs were observed on the basis of reciprocal BLAST search among genus *Eucalyptus* including 13 repeat units identified only in a particular species (supplementary file 1).

Identified repeating units were not equally present in all cp-genomes of genus *Eucalyptus*, therefore, the frequency of common and putative polymorphic cpSSRs varied between species. Previously, distribution and organization of cpSSRs were observed in *Eucalyptus* spp (30), however, the information about length variation of cpSSRs between species was not explored.

The variation in frequency of common and polymorphic cpSSRs between species was in conformity with genus *Arabidopsis* (19). Frequency of common, putative polymorphic and unique cpSSRs identified between species is shown in Supplementary table 1. Details of identified putative polymorphic and common cpSSRs for each species of *Eucalyptus* considered are given in supplementary file 2 and partially illustrated with reference to *E. globulus* in Fig. 2.

Transferability of common and polymorphic cpSSRs in genus Eucalyptus

E. globulus was considered as reference species to design PCR primer pairs and to *in silico* amplify PCR product. Details of designed primer pairs of cpSSRs motif, their length, start-end position, forward/reverse primer sequences, primer length, annealing temperature, GC content and product size are presented in Table 3. There are reports on the transferability of SSRs within a genus can vary (38). In the present study, most of the cpSSRs primer pairs from reference species showed higher transferability rate, ranged from 93.54% to 100%, among the genera. This is due to higher conservation of chloroplast genome sequences in different species of *Eucalyptus*.

Table 1. List of chloroplast genomes of *Eucalyptus* along with their sequence accession number

Sl. No.	Organism	Abr	Size (Kb)	Accession No.	References
1.	*Eucalyptus aromaphloia*	*Ea*	160.149	NC_022396.1	(41)
2.	*Eucalyptus baxteri*	*Eb*	160.032	NC_022382.1	(41)
3.	*Eucalyptus camaldulensis*	*Eca*	160.164	NC_022398.1	(41)
4.	*Eucalyptus cladocalyx*	*Ecl*	160.213	NC_022394.1	(41)
5.	*Eucalyptus cloeziana*	*Eco*	160.015	NC_022388.1	(41)
6.	*Eucalyptus curtisii*	*Ecu*	160.038	NC_022391.1	(41)
7.	*Eucalyptus deglupta*	*Edg*	160.177	NC_022399.1	(41)
8.	*Eucalyptus delegatensis*	*Edl*	159.724	NC_022380.1	(41)
9.	*Eucalyptus diversicolor*	*Edc*	160.214	NC_022402.1	(41)
10.	*Eucalyptus diversifolia*	*Edf*	159.954	NC_022383.1	(41)
11.	*Eucalyptus elata*	*Eel*	159.899	NC_022385.1	(41)
12.	*Eucalyptus erythrocorys*	*Eer*	159.742	NC_022406.1	(41)
13.	*Eucalyptus globulus*	*Egl*	160.286	NC_008115.1	(42)
14.	*Eucalyptus grandis*	*Egr*	160.137	NC_014570.1	(43)
15.	*Eucalyptus guilfoylei*	*Egu*	160.520	NC_022405.1	(41)
16.	*Eucalyptus marginata*	*Ema*	160.076	NC_022390.1	(41)
17.	*Eucalyptus melliodora*	*Eme*	160.386	NC_022392.1	(41)
18.	*Eucalyptus microcorys*	*Emi*	160.225	NC_022404.1	(41)
19.	*Eucalyptus nitens*	*En*	160.271	NC_022395.1	(41)
20.	*Eucalyptus obliqua*	*Eo*	159.527	NC_022378.1	(41)
21.	*Eucalyptus patens*	*Epa*	160.187	NC_022389.1	(41)
22.	*Eucalyptus polybractea*	*Epo*	160.268	NC_022393.1	(41)
23.	*Eucalyptus radiata*	*Era*	159.529	NC_022379.1	(41)
24.	*Eucalyptus regnans*	*Ere*	160.031	NC_022386.1	(41)
25.	*Eucalyptus saligna*	*Es*	160.015	NC_022397.1	(41)
26.	*Eucalyptus salmonophloia*	*Esa*	160.413	NC_022403.1	(41)
27.	*Eucalyptus sieberi*	*Esi*	159.985	NC_022384.1	(41)
28.	*Eucalyptus spathulata*	*Esp*	161.071	NC_022400.1	(41)
29.	*Eucalyptus torquata*	*Et*	160.223	NC_022401.1	(41)
30.	*Eucalyptus umbra*	*Eu*	159.576	NC_022387.1	(41)
31.	*Eucalyptus verrucata*	*Ev*	160.109	NC_022381.1	(41)

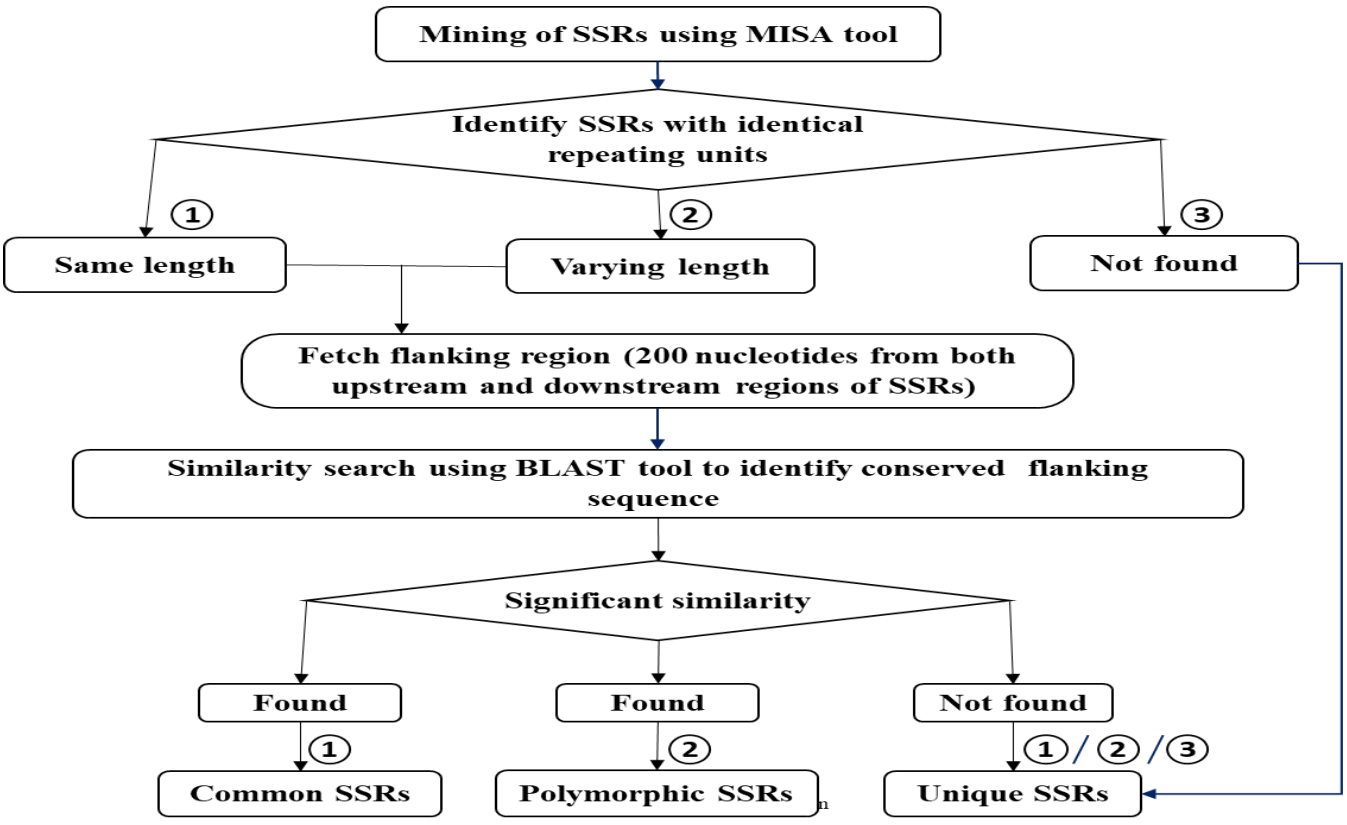

Fig. 1. Flowchart to identify common, unique and putative polymorphic SSRs.

Table 2. List of SSR motifs identified in chloroplast genomes of *Eucalyptus* species along with their frequency.

Sl. No.	SSR motif	No. of species	Frequency in genomes
Identical repeating units with varying length			
1.	A	31	209
2.	AT	15	16
3.	ATAA	30	30
4.	T	31	182
5.	TTA	19	19
6.	TTTC	31	61
Identical repeating units with same length			
1.	AAATG	5	5
2.	AAT	3	3
3.	AATA	31	32
4.	ATAG	31	58
5.	ATT	27	28
6.	ATTA	30	45
7.	ATTT	26	27
8.	CTT	31	31
9.	CTTG	31	31
10.	GAA	30	30
11.	GATA	3	3
12.	TAAG	31	31
13.	TAAT	20	35
14.	TATTT	2	2
15.	TTAA	29	29
Unique repeating units			
1.	AAAT	1	1
2.	AATT	1	1
3.	CCTGAG	1	1
4.	CTATA	1	1
5.	CTTT	1	1
6.	G	1	1
7.	GAAAGG	1	1
8.	GGCAT	1	1
9.	TAT	1	1
10.	TATT	1	1
11.	TATTC	1	1
12.	TTC	1	1
13.	TTCCAT	1	1

Putative polymorphic cpSSR motifs, with length variation, A/T showed 96.77% transferability rate among genus *Eucalyptus* (Fig. 3). Motif A was not amplified in *E. cloeziana* and *E. erythrocorys*, whereas motif T was not amplified in *E. obliqua* and *E. radiata.*

Among common cpSSRs, PCR amplification of same length motifs AATA (100%) showed higher transferability rate followed by GAA (93.54%) among *Eucalyptus* (Fig. 4). Motif GAA was not amplified in *E. diversicolor* and *E. marginata*, whereas, AATA showed transferability with every species among the genus *Eucalyptus.*

Earlier, many of the SSR loci isolated from *E. grandis* and *E. urophylla* were cross amplified with other species of *Eucalyptus* (39). Gene-homologous SSRs designed for *E. gomphocephala* were successfully transferred to *E. marginata, E. camaldulensis* and *E. victrix* (40). The present work showed higher transferability in comparison to four nuclear SSRs (50%) from *E. nitine* to sub genera *Symphyomyrtus* and *Monocalyptus* (9). Moreover, 60 primer pairs were developed and virtually amplified among genus *Eucalyptus* displayed interspecific polymorphism (30). Recently, 12 and 11 markers of *Lasiodiplodia mahajangana* and *Neofusicoccum parvum* tested for length variation and high transferability of the developed markers were observed (34).

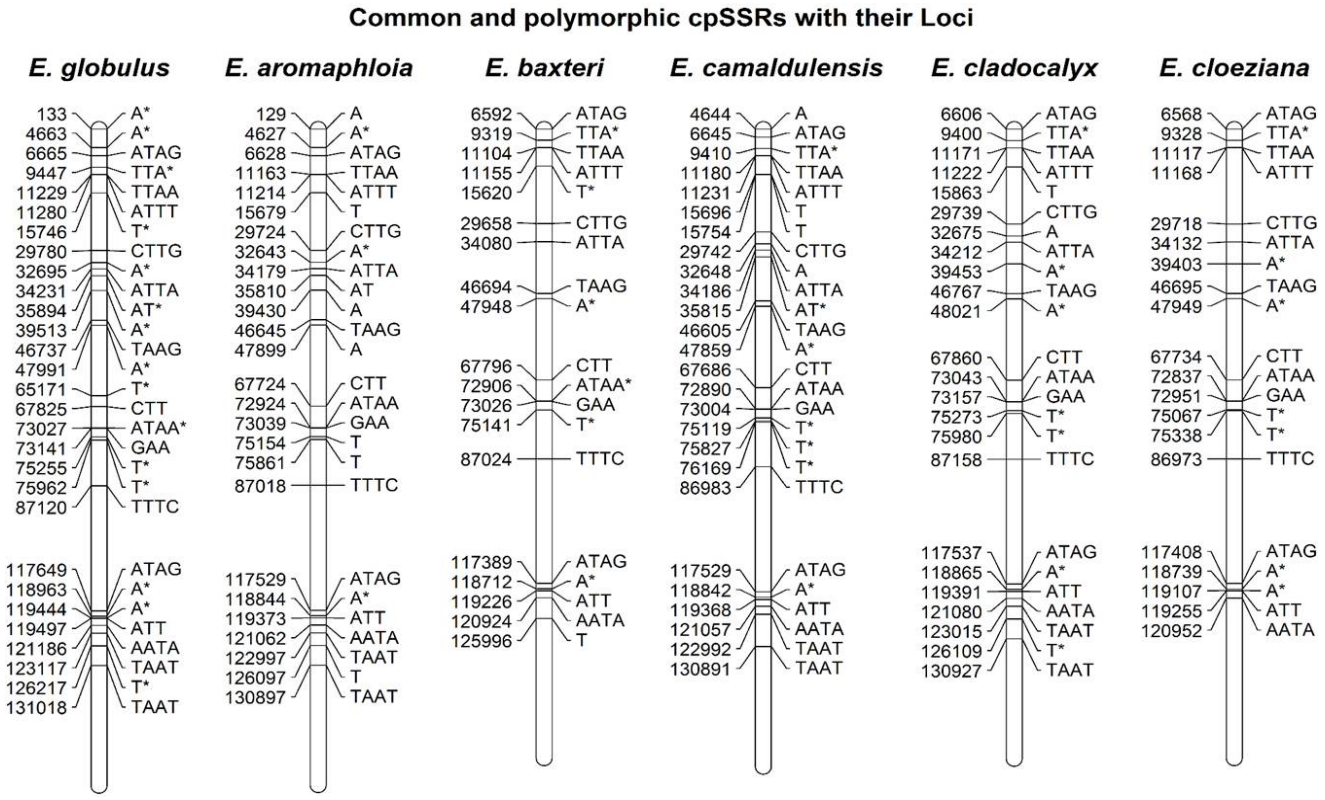

Fig. 2. Loci of common and polymorphic (indicated with *) cpSSRs along with their motif and start position between *Eucalyptus globulus*, as reference and other *Eucalyptus* spp. Organisms' abbreviations are given in Table 1. Full figure is given as Supplementary Fig. 1.

Table 3. Information of SSR motifs and primers used for *in silico* PCR

Sl. No.	Motif	Motif Length	Star/ End	Forward / Reverse Primer	Primer Length (bp)	Temp	GC %	Product Size
Motifs with varying length among genome								
1.	A	13	133 145	AATCCACTGCCTTGATCCAC GAAAGGTTATAATTTTCTGCTTCTTC	20 26	59.93 57.48	50.00 30.77	227
2.	T	12	15746 15757	CGTTCTGCTTGGCCCATTTAATTT AGGTCGAATAATTGAATAGCA	25 20	60.68 59.08	36.00 40.00	246
Motifs with same length among genome								
1.	AATA	12	121186 121197	CCATAGAACATTTGGCGTAACA GAATGGCTATCCACCTTCCA	22 20	59.89 59.89	40.91 50.00	232
2.	GAA	12	73141 73152	CCCAAATAAAAATCCGAACTCA ATGGAGTTCGCTGGAGAATG	22 20	60.17 60.22	36.00 36.50	203

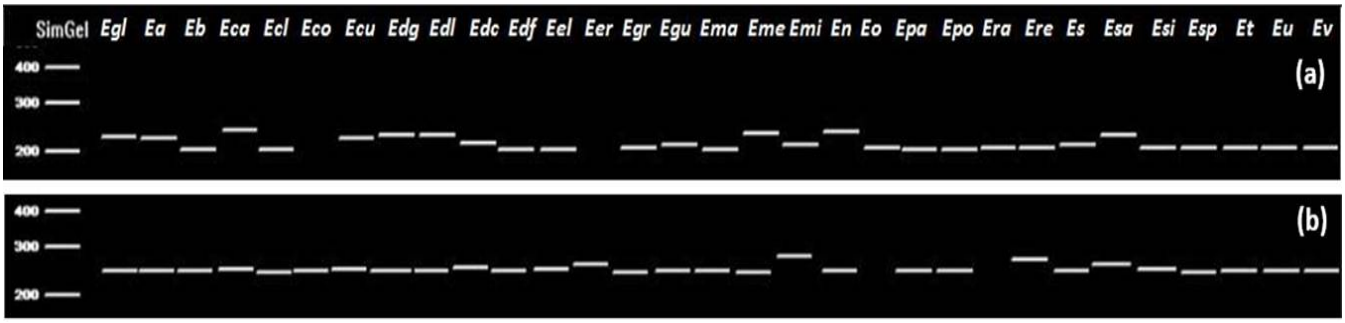

Fig. 3. *In silico* transferability of cpSSRs with varying length motifs (a) A and (b) T. Organisms' abbreviations are given in Table 1.

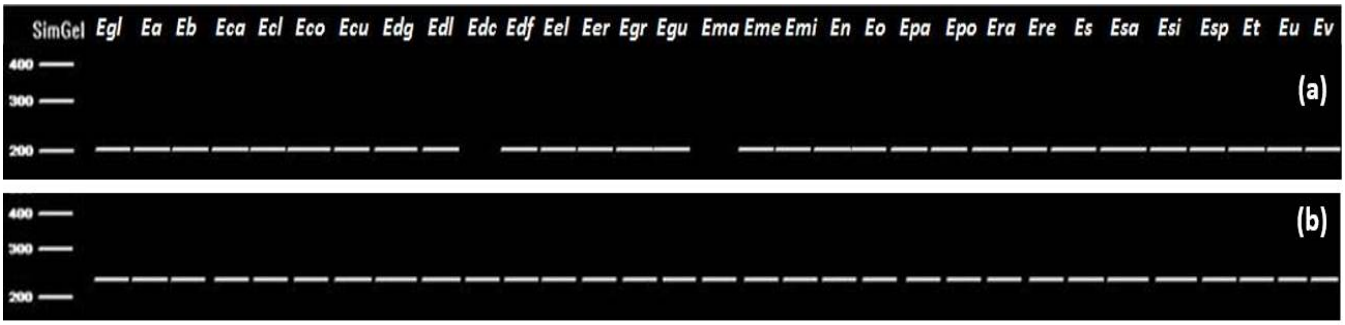

Fig. 4. *In silico* transferability of cpSSRs with same length motifs, (a) GAA and (b) AATA. Organisms' abbreviations are given in Table 1.

Conclusion

Distribution of common, unique and polymorphic cpSSRs were successfully analyzed among the genus *Eucalyptus*. Moreover, cross-species transferability of selected common and putative polymorphic cpSSRs were studied among genus *Eucalyptus*. The present study will play an important role in species identification, phylogeny, genetic diversity studies, genetic mapping etc. and will surely enhance the utility and potential of cpSSRs in *Eucalyptus* and other related genera.

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Authors' contributions

SK performed the work and drafted the manuscript. AS designed the study framework and helped in the analysis of the data generated.

Conflicts of interests

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

Supplementary files

Supplementary File 1: Details of identified unique cpSSRs among genus *Eucalyptus.*

Supplementary File 2: Details of identified putative polymorphic and common cpSSRs for each species of *Eucalyptus.*

Supplementary Table 1: Frequency of common, unique (*U*), and putative polymorphic (bold) cpSSRs identified in genus *Eucalyptus*. Organisms' abbreviations are given in Table 1.

Supplementary Fig. 1: Loci of common and polymorphic (indicated with *) cpSSRs (with their start position) between reference (*E. globulus*) and other species.

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