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Original Article

MOLECULAR CHARACTERIZATION OF ENDANGERED MEDICINAL PLANT SPECIES *HEDYCHIUM CORONARIUM* FROM EASTERN INDIA

REENA PARIDA[1]*, SUJATA MOHANTY[1], SANGHAMITRA NAYAK[1]

Centre of Biotechnology, Siksha O Anusandhan University, Kalinga Nagar, Ghatikia, Bhubaneswar-751003, Odisha, India
Email: ms.reenas@gmail.com

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ABSTRACT

Objective: Molecular characterization of *Hedychium coronarium* from 4 different populations of Odisha using 9 inter simple sequence repeats and 15 random amplified polymorphic DNA markers to indicate the closeness of species and hybrids quickly and efficiently.

Methods: A dendrogram was constructed through sequential agglomerative hierarchial and nested (SAHN) clustering and un-weighted pair group method with arithmetic mean (UPGMA) analysis using Jaccard's similarity coefficient of combined markers using this particular species.

Results: Two major clusters were found, i.e., cluster-I (Malkangiri-1, Phulabani-1, Phulabani-3, Malkangiri-2, Khurda-1, Khurda-2, Khurda-3, Angul-3, Angul-1, Angul-2 and Phulabani-2) and cluster-II (Malkangiri-3). The clustering pattern also revealed moreover the extent of genetic similarity between germplasms collected from four different regions population.

Conclusion: The potential of this technique would be further realised to the fullest extent for the identification and tagging of the important novel gene in different taxa, unexplored yet, thus facilitating the improvement of desired taxa of Zingiberaceae. The findings would be of immense enough significance for complementing the strategies of conservation and characterization of these important taxa of Zingiberaceae following modern biotechnological approach.

Keywords: *Hedychium coronarium*, ISSR, RAPD, Molecular characterization.

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INTRODUCTION

There are several molecular markers which have been regularly used for studying genetic relations, population genetics and genetic characterization in different plant groups and crop cultivars. The molecular markers are not influenced by the external environmental factors unlike that of morphological markers hence accurately testify genetic relationship between and among plant groups. Also for proper conservation programme characterization of plants genetically is necessary. These are now routinely used as because it is more reliable, less time consuming and easy to handle in comparison with morphological and biochemical markers. It helps to indicate the closeness of species and hybrids quickly and efficiently [1]. Now days, number of molecular markers (random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), inter-simple sequence, repeats (ISSR), simple sequence repeats (SSR), isozymes, proteins, etc.) are being widely used for germplasm evaluation, measuring genetic diversity, genetic mapping, for assessing genetic relationship among different taxa and for the detection of genetic changes caused due to mutation or genetic engineering. RAPD have been used for measuring genetic diversity in several plant species like apple [2], wheat [3], *Piper* [4], *Triticum* [5], *Gossypium* [6], *Oryza* [7], Barley [8], Sugarcane [9], *Cymbopogon* [10], Tea [11], Soybean [12], Banana [13], Broccoli [14]. DNA markers are widely used in genome mapping in a wide range of plant species and are now being increasingly employed for studies of genetic relationship among species and within accessions [15, 16]. RAPD and AFLP (amplified fragment length polymorphism) markers have the potential to reveal a large amount of variation with good coverage of entire genome [17, 18]. There are many reports available on genetic and chromatographic fingerprinting of medicinal and aromatic plants for identification [19-23]. Some reported ISSR as a valuable tool for genetic diversity analysis in spices [24]. The competence of ISSR in clonal fidelity assessment on *Allium* and *Aloe* was established successfully [25]. The genetic fidelity of *vanilla* using RAPD and ISSR primers [26]. More recently [27] used RAPD and ISSR markers in vanilla to assess the genetic diversity and [28] used ISSR marker to determine the level of genetic diversity and relatedness among strawberry cultivars. Previously there was no report about the molecular characterization of *Hedychium coronarium* from eastern India. This molecular technique could be used in the study of genetic diversity of endangered plant species to conserve the particular species.

MATERIALS AND METHODS

Plant material

In the present investigation, *Hedychium coronation* was collected from the different wild areas of Odisha like Malkangiri, Phulabani, Khurda and Angul districts. After collection, the rhizomes of these medicinal plant samples were grown in the medicinal plant garden of Center Of Biotechnology, Siksha O Anusandhan University, Bhubaneswar, Odisha.

Isolation of genomic DNA

Genomic DNA was isolated by following the protocol of [29] with little modification. Two grams of fresh, young leaf samples was grinded with 2% insoluble PVPP to make a fine powder in a cold mortar and pestle with repeated addition of liquid nitrogen. Thawing was avoided to reduce the shearing of DNA. The powder was then transferred to a 50 ml centrifuge tube containing 10 ml of pre-warmed (60°C) 2% CTAB-DNA extraction buffer (10% CTAB; 4M NaCl; 0.5M EDTA, pH 8; 1M Tris-HCl, pH 8; 2% β-mercaptoethanol) and was mixed vigorously. The mixture was incubated in a water bath (YSI412, Yorco universal) for one hour at 65°C with intermittent gentle shaking. After incubation, the mixture was cooled to room temperature and emulsified with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and was gently mixed. Then, it was centrifuged at 10,000 rpm for 20 min in a cooling centrifuge (C-24BL, Remi) at room temperature. The upper aqueous phase was pipetted out with the help of a micropipette into another 50 ml centrifuge tube and mixed with 2.5 volume of pre-chilled dehydrated ethanol. After quick inversion, DNA, like a mass

of cotton threads was precipitated. The precipitated nucleic acid was spooled out with a bend glass Pasteur pipette, washed twice with 70% ethanol, stored in a 1.5 ml microcentrifuge tube and dried. The dried DNA was dissolved in an excess amount of $T_{10}E_1$ buffer (Tris-Cl 10 mmol, EDTA 1 mmol with pH 8).

Purification of genomic DNA

The dissolved DNA was impure with proteins, RNA and phenolic in some cases so the crude DNA was purified and RNA was removed. The RNA was removed by giving RNase treatment. For 1 ml of crude DNA solution, 60 µg of RNase A was added, and the solution was incubated with continuous shaking in a water bath at 37 °C for 1 hr. After one hour it was removed from the water bath and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and gently mixed thoroughly. The solution was then centrifuged in cooling centrifuge at 10,000 rpm for 20 min at 20 °C and the upper aqueous phase was pipetted out. It was again washed with chloroform: isoamyl alcohol (24:1) twice and centrifuged at 10,000 rpm for 20 min at room temperature. The upper aqueous phase was separated after centrifugation (as described earlier) and mixed with $1/10^{th}$volume of 3M sodium acetate (pH 4.8). DNA was precipitated by adding 2.5 volume of chilled absolute ethanol and pelleted by spinning. The pellet was washed twice with 70 % ethanol, carefully and dried under vacuum. The dried DNA was dissolved in minimum amount of $T_{10}E_1$ buffer (pH 8).

Test for quality and quantity of the purified DNA

The quality and quantity of DNA were measured by UV-vis spectrophotometer (Model evolution 220, Thermo Fisher Scientific,). The total DNA quantity was measured by taking the absorbance at 260 nm wavelength and the quality of the DNA was confirmed from the absorbance ratio at 260 nm and 280 nm. It was reported that if the ratio is about 1.8 to 2.0 then the quality of the DNA is good [30]. For final checking the quality as well as the quantity of DNA, the DNA was loaded in 0.8% agarose gel alongside diluted uncut lambda DNA as standard and electrophoresed. It was observed that the DNA from all the samples was very good in quality. After quantification, the DNA was diluted with $T_{10}E_1$ buffer to a working concentration of 25-ng/µl for RAPD and ISSR analysis.

RAPD analysis

Two types of polymerase chain reaction (PCR) based molecular techniques namely RAPD and ISSR were utilised for the present study.

For RAPD analysis random decamer operon primers were dissolved in double sterilised $T_{10}E_1$ buffer, pH 8.0 to the working concentration of 15 ng/µl. Few selected primers as per the reproducibility and amplification pattern from A, C, D, N and AF series OPA04, OPA07, OPA09, OPA18, OPC02, OPC05, OPC011, OPD03, OPD07, OPD08, OPD12, OPD18, OPD20, OPN04, OPN16, OPN18, AF5, AF14 and AF15, etc. were used for RAPD analysis. The RAPD analysis was performed as per the methodology described by [31]. Each amplification reaction mixture of 25 µl volume contained 2.5 µl of 10X assay buffer (100 mmol Tris-HCl, pH 8.3, 500 mmol KCl, 1.5 mmol $MgCl_2$ and 0.1% gelatin), 200 µM of each dNTPs (dATP, dCTP, dGTP and dTTP), 15 ng of primer, 0.5 unit of Taq DNA polymerase and 25 ng of template DNA. The amplification reaction was carried out in PCR (Gene amp PCR system 9700, Applied Biosystems). The amplification was performed in three steps PCR. The initial denaturation of the template DNA was carried out at 94 °C for 5 min for one cycle. The second step was carried out for 42 cycles and each cycle consisting of three temperature steps, i.e., one minute at 92 °C for denaturation of the template, one minute at 37 °C for primer annealing followed by two minutes at 72 °C for primer extension. The final step consisted of only one cycle i.e. 7 min at 72 °C for complete polymerization. The soaking temperature was 4 °C. After the completion of the PCR 2.5 µl of 6X loading dye was added to the amplified products and was stored at–20 °C till further use.

ISSR analysis

Nine numbers of ISSR primers were used for ISSR analysis. Those primers were namely $(GAC)_5$, $(GTGC)_4$, $(GACA)_4$, $(AGG)_6$, $(GA)_9T$, $T(GA)_9$, $(GTG)_5$, $(GGA)_4$ and $(CAA)_5$. The ISSR analysis was performed as per the methodology is given by [32]. Each amplification reaction mixture of 25 µl contained 25 ng of template DNA, 2.5 µl of 10X assay buffer (100 mmol Tris-HCl pH 8.3, 500 mmol KCl, 1.5 mmol $MgCl_2$ and 0.1% gelatin), 200µM each of dNTPs (dATP, dCTP, dGTP and dTTP), 44 ng of primer and 0.5 unit Taq DNA polymerase. The amplification was carried out in a thermal cycler. The first cycle consisted of denaturation of template DNA at 94 °C for 5 min, primer annealing at a specific temperature for a particular primer for 1 minute and primer extension at 72 °C for 2 min. In the subsequent 42 cycles, the period of denaturation was reduced to 1 minute while the primer annealing and primer extension time were maintained same as in the first cycle. The last cycle consisted of only primer extension at 72 °C for 7 min and then the amplified products were resolved in 2% agarose gel stained with ethidium bromide.

Agarose gel electrophoresis

The PCR products for RAPD were separated in 1.5% agarose gel while those of the ISSR products were resolved in 2% agarose gel. The agarose gel was prepared with TAE buffer (40 mmol Tris base, 20 mmol sodium acetate, 20 mmol EDTA, glacial acetic acid; pH 7.2), 125 ml 1X TAE buffer was taken in a 500 ml conical flask and 1.875 gm of agarose was added, boiled for complete melting of agarose and then cooled to 50 °C. After cooling, 6.25 µl of ethidium bromide solution (10 mg/ml) was added, mixed properly and the gel solution was poured on the gel casting tray and left for one hour for complete gelling. Then the gel was submerged in a gel tank (1704495 Sub-Cell Model, Bio-Rad) containing 1X TAE buffer. Prior to loading the samples, the comb was removed. In the submerged gel 27 µl of the PCR amplified samples containing the tracking dye were loaded in each well. Standard DNA ladder (Gene Ruler 100 bp) was loaded in the first well after the amplified samples were loaded in other wells to know the size of the amplified DNA fragment. The electrophoresis was performed at 60 volts for 3 h.

After electrophoresis, the gel was visualised under the UV-trans illuminator (0507, BioRad) and photographed using gel documenting system (76S/07634, Universal Hood II Bio-Rad) for scoring the bands. The sizes of the amplicons were determined by comparing them with that of the ladder. The entire process was repeated at least twice to confirm the reproducibility.

Scoring of the data

The data was scored as '1' for the presence and '0' for the absence of the band for each primer genotype combination for RAPD and ISSR analysis. All the bands were considered underestimation of the genetic similarity [33].

Statistical analysis of the data

Resolving power (Rp)

Resolving power of the RAPD and ISSR were calculated as per [34]. Resolving power is: Rp=ΣIB (IB (Band informative-ness) = 1-[2×(0.5-P)], P is the proportion of the species containing the band.

Primer index (PI)

The primer index was calculated from the polymorphic index. A polymorphic index (PIC) was calculated as PIC = $1-\sum P^2_i$, P_i is the band frequency of the *i* th allele [35]. In the case of RAPDs and ISSRs, the PIC was considered to be $1-p^2-q^2$, where p is band frequency and q is no band frequency [36]. The PIC value was then used to calculate the primer index (PI). PI is the sum of the PIC of all the markers amplified by the same primer.

Jaccard's similarity

Jaccard's coefficient of similarity [37] was measured and a phylogram based on similarity coefficients was generated by un-weighted pair group method using arithmetic averages (UPGMA) [38] and the SAHN clustering was obtained. The entire analysis was performed using the statistical package NTSYS-pc 2.02e [39].

Bootstrapping

In addition to the classical resampling methods the statistical testing of robustness of the obtained trees, such as bootstrapping was

implemented. This test has been created to give a possibility of having more distances with the same values. In such case, the order of taxa influences the result of the tree building. Rearrangement of taxa could reveal this situation.

Chemicals and reagents:

RNase A (Qiagen Inc., USA)

Agarose (SRL Pvt. Ltd, India)

RAPD operon primers (Operon Tech., Alameda, USA)

dNTP (MBI Fermentas, Lithuania)

Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India)

6X loading dye (MBI Fermentas, Lithuania)

ISSR primers (Bangalore Genei Pvt. Ltd, Bangalore, India)

DNA ladder (MBI Fermentas, Lithuania)

RESULTS

Genomic DNA isolation and quantification

The modified CTAB protocol yielded a good quality of DNA as revealed by agarose gel electrophoresis. The concentration of DNA varied from 100-350 ng/µl.

RAPD analysis

Out of 25 random decamer oligonucleotide primers, 15 primers were used for the present work basing upon their amplification and clarity of banding pattern. All the 4 populations of *H. coronarium* (Malkangiri, Phulabani, Khurda, Angul), each in triplicates, were used which produced distinct, reproducible amplicons (fig. 1a, b). A total of 62 bands were amplified all of which were found to be monomorphic in nature. The highest number of bands (7) were amplified with primer OPD20 (450-3000bp), and lowest number of the band (2) was amplified with primer OPA18 (1850-2000bp). No unique bands were found with all the primers. An average number of bands per primer was found to be 4.1. The resolving power of the primers was varied from 4-14 where the primer with maximum resolution power was OPD20 (14) and the primer with minimum resolution power was OPA18 (4) (table 1).

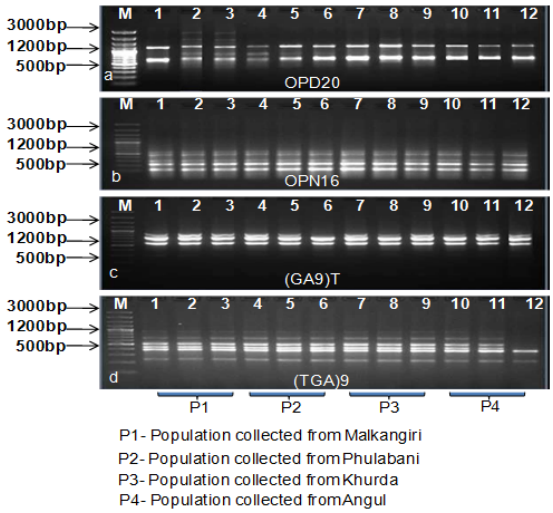

Fig. 1(a, b) RAPD banding pattern (c, d) ISSR banding pattern of *Hedychium corornarium* from different populations (Lane 1-12) and M–marker

Table 1: Details of RAPD, ISSR and combined marker analysis as revealed among 4 populations of *Hedychium corornarium*

Markers	Primer	Sequence of Oligonucleotides	Approx fragment Size(bp)	Total bands	Monomorphic bands	Polymorphic bands	Unique bands	Resolving power
	OPA4	5'AATCGGGCTG3'	600-1400	5	5	0	0	10
RAPD	OPA7	5'GAAACGGGTG3'	400-2200	4	4	0	0	8
	OPA8	5'AGGTGACCGT3'	750-1350	4	4	0	0	8
	OPA18	5'AGGTGACCGT3'	1850-2000	2	2	0	0	4
	OPC2	5'GTGAGGCGTC3'	600-1300	3	3	0	0	6
	OPC5	5'GATGACCGCC3'	900-2000	5	5	0	0	10
	OPC11	5'AAAGCTGCGG3'	600-1500	5	5	0	0	10
	OPD3	5'GTCGCCGTCA3'	1031-1600	3	3	0	0	6
	OPD7	5'TTGGCACGGG3'	400-2000	4	4	0	0	8
	OPD8	5'GTGTGCCCCA3'	900-1800	4	4	0	0	8
	OPD18	5'GAGAGCCAAC3'	225-1500	3	3	0	0	6
	OPD20	5'ACCCGGTCAC3'	450-3000	7	7	0	0	14
	OPN4	5'GACCGACCCA3'	500-1650	5	5	0	0	10
	OPN16	5'AAGCGACCTG3'	250-400	4	4	0	0	8
	OPN18	5'GGTGAGGTCA3'	450-1400	4	4	0	0	8
Total				**62**	**62**	**0**	**0**	
ISSR	SPS1	(GAC)5	280-250	13	13	0	0	26
	SPS2	(GTGC)4	325-000	8	8	0	0	16
	SPS3	(GACA)4	325-1350	10	6	4	2	15
	SPS4	(AGG)6	275-825	9	9	0	0	18
	SPS5	(GA)9T	300-850	10	10	0	0	20
	SPS6	T(GA)9	225-950	6	6	0	0	12
	SPS7	(GTG)5	180-1200	10	10	0	0	20
	SPS8	(GGA)4	300-885	7	3	4	4	6.666
	SPS9	(CAA)5	550-1600	5	4	1	0	9
Total				**78**	**69**	**9**	**6**	**6**
Grand total				**140**	**131**	**9**	**6**	

ISSR analysis

9 ISSR primers resulted in the amplification of 78 fragments. The primer (GAC) 5 produced the maximum number of bands (13), while primer (CAA) 5 produced a minimum number of bands (5). From 78 bands amplified, 69 bands were monomorphic, 3 were polymorphic and only 6 were found to be unique bands. The bands were amplified in the range of 180-2000bp. Among these ISSR primers,

maximum resolving power (26) was obtained in (GAC)5 primer and minimum Rp (6.666) was in (CAA)5 (table 1, fig. 1c, d).

Analysis of data of combined markers

For assessment of genetic similarity among 4 populations of *H. coronarium,* two types of marker combination (RAPD and ISSR) was taken. A total of 140 bands were amplified with all the marker out of which 131 were monomorphic, 3 were polymorphic and rest 6 were unique bands (table 1). All the samples were correlated with each other with an average similarity of 0.978 which ranged 0.957 to 1.000. The dendrogram constructed using Jaccard's similarity coefficient, separated the 4 populations into two major clusters, one with 11 samples another with rest 1 sample at similarity coefficient of 0.96 (fig. 2). Cluster II included one replicate of Malkangiri population while cluster I included rest of the populations.

Cluster I further divided into two subgroups which include three replicates of Angul and Khurda populations, two replicates each of Malkangiri and Phulabani populations while subcluster B includes only one replicate of Phulabani population.

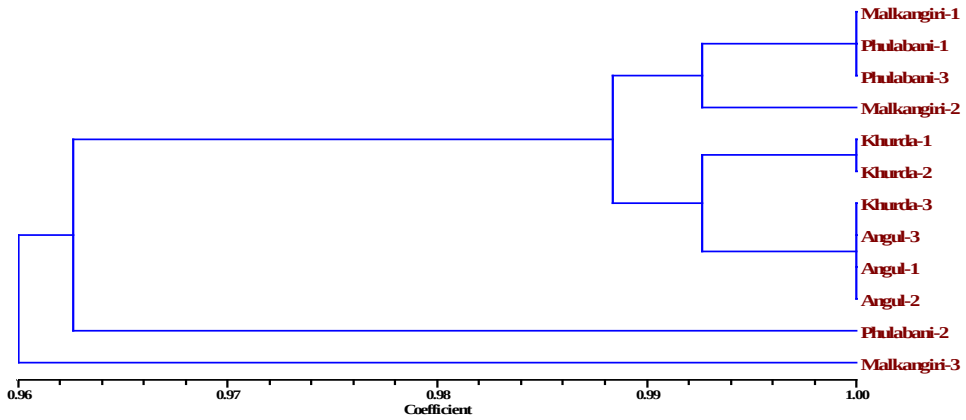

Fig. 2: Dendrogram showing genomic relationship within 4 populations in *H. coronarium* as revealed from combined RAPD and ISSR analysis

DISCUSSION

Accurate identification and characterization of different germplasm resources is important for species identification, cultivar development, certification and breeder's right's protection [40, 41]. With the advent of molecular biology techniques, DNA-based markers very efficiently augment morphological, cytological, and biochemical characters in germplasm characterization, varietal identification, clonal fidelity testing, assessment of genetic diversity, validation of genetic relationship, phylogenetic and evolutionary studies, marker-assisted selection and gene tagging. Owing to plasticity, ubiquity and stability, DNA markers are easier, efficient, and less time consuming especially in perennials where morphological markers are few. In recent times there is an increased emphasis in molecular markers for identification and characterization of the genotypes, genetic fingerprinting in identification and cloning of important genes, marker-assisted selection and in the understanding of interrelationships at the molecular level. The polymerase chain reaction (PCR), based technologies such as RAPD, ISSR, AFLP and microsatellites, are widely appreciated for its genetic integrity as well as cost effectiveness and simplicity. Although work on morphological characterization of Zingiberaceous species has been attempted, its molecular characterization is still in a nascent stage except for some genetic fidelity studies of micro-propagated plants and isozyme-based characterization [15, 16, 42].

The origin and relationships of *Alpinia galanga* based on its molecular data were studied by [43]. RAPD markers were used to assess the level of genetic diversity in 71 *Alpinia* accessions (31 cultivated and 6 wild) collected from different areas of Thailand [44]. Their UPGMA cluster analysis of genetic similarity also separated the accessions into 5 major clusters. Molecular genetic fingerprints of nine *Curcuma* species from Northeast India using PCR-based markers were studied by [45]. A phylogenetic analysis of the tribe Zingiberaceae was performed by [46] using nuclear ribosomal DNA (ITS1, 5.8S, and ITS2) and chloroplast DNA (trnL [UAA] 5′ exon to trnF [GAA]). The study indicated that tribe Zingiberaceae is monophyletic with two major clades, the *Curcuma* clade, and the *Hedychium* clade. The general *Boesenbergia* and *Curcuma* are apparently not monophyletic. The populations having similarity within each other is grouped under the same cluster which is similar with the report of [47]. In his study of genetic diversity among ginger germplasm collected from gene bank, local farm and market collection and found one accession from Bangladesh to be identical with one accession from Pakistan and two accessions each from Japan and China were also similar to each other. He explained the occurrence of similarity could be the presence of the same genotype in the market in different countries which matches with our result and with our supposition as presented above. Some other studies like gas chromatography-mass spectrometry (GC-MS) and high-performance thin layer chromatography (HPTLC) analysis could be done for identification of elite cultivars [48, 49].

CONCLUSION

It gives a good control on the preservation of genetic resources and facilitates international exchanges of healthy plant material. In the present report, two PCR-based molecular markers like RAPD and ISSR has been used to characterise different populations of Zingiberaceae. Basing upon their genetic similarity dendrograms were constructed for each species which confirm their genetic relationship by dividing the populations into many clusters and subclusters. Samples present in single clusters represent their closeness with each other.

Hence it could be concluded that there must be an intraspecific polymorphism in between the populations. At the same time, less number of samples analysed need a further classification. The potential of the present work will be realised to the fullest extent for the establishment of relationship within a population of Zingiberaceae.

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CONFLICT OF INTERESTS

Declared none

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