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Analysis of Genetic Diversity among *Brassica juncea* Genotypes using Morpho-physiological and SSR Markers

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A B S T R A C T

Indian mustard has the share of over 80% to the total rapeseed-mustard production in the country. Present investigation is conducted to guesstimate the possible presence of diversity among 38 germplasm lines and 5 cultivated varieties of Indian mustard by using 12 morpho-physiological parameters as well as microsatellite markers. Phenotypic analysis of variance for 12 yield and its attributing traits revealed that, the genotypes taken for this investigation had significant amount of genetic variation. Variances due to genotypes were highly significant for all the characters except numbers of primary branches per plant, siliqua length, numbers of seeds/siliqua and 1000-seed weight. In our study, a total of eleven polymorphic SSR molecular markers were used and showed sufficiently high sensitivity to detect DNA polymorphisms. Information generated from the current study would be helpful in the improvement of Indian mustard by the means of conventional and/or molecular breeding in impending days.

Introduction

Brassica juncea is the valuable oilseed crop after soybean and palm oil. It is also considered as the second largest source of protein after soybean and consumption of each part of plant by human as well as animals. Due to certain other qualities of mustard seeds which contain 30-46% oil with good amount of protein content (17-22.0%), the oil became popular in India. Indian mustard is also an important component in the oilseed sector of the country. Indian people consume *B. Juncea* in the form of oil however it is also important for livestock feed. Oil consumption has increased due to budding population and improving life standards. So, the present oil requirements can be fulfilled through increment in the yield potential of *B. juncea* using breeding approaches. Genetic diversity decides the use of two parents for hybridization purposes.

Genetic assortment allows the populations to adapt in changing environment. The basic exercise of the evaluation of inherent

variability in a particular crop species is required to start any breeding programme. An array of techniques is available now days for the identification of variability in crop plants including morpho-physiological, biochemical and molecular markers (Mohammadi and Prasanna, 2003).

Reports are also available on the assessment of diversity in *B. juncea* using different approaches (Vaishnava *et al.,* 2006; Alie *et al.,* 2009; Shyam and Tripathi, 2019; Shyam *et al.,* 2020).

Amongst the diverse types of molecular markers employed, microsatellites are advantageous due to their higher reproducibility, co-dominance nature and abundance, wide distribution throughout the genome, easy storability and multi-allelic variation (Powell *et al.,* 1996). The strengths of SSR markers are by reason of its presence in soaring numbers in eukaryotes. Furthermore, it can be functional even with low quantities of template DNA.

In addition to that, it is an improved technique which is simpler and more efficient to find polymorphism (Tiwari *et al.,* 2019) but some of the breeding groups advocate morphological traits than molecular markers (Hu *et al.,* 2007; Kachare *et al.,* 2019; Mishra *et al.,* 2020; Upadhyay *et al.,* 2020) as a result, present investigation is carried to estimate the diversity present among thirty-eight germplasm lines and 5 cultivated varieties of Indian mustard at molecular level and to assess the genetic relationship within and between genotypes by using 12 different morpho-physiological parameters as well as SSR molecular markers.

The intent was to combine the relative efficiency of both approaches to lead into a more informative result for characterization of genotypes of *B. juncea*.

Materials and Methods

Experimental materials

A total of 38 germplasm lines and 5 cultivated varieties (Table 1) of *B. juncea* were preferred for phenotypic and molecular analysis. These germplasm lines are currently being cultivated in Morena district and various adjoining regions of Gwalior district of Madhya Pradesh and some of them are also cultivated in different agro-climatic zones across India. The seed of germplasm lines were collected from Zonal Agricultural Research Station, Morena, Rajmata Vijayaraje Scindia Agricultural University, Gwalior, India.

Phenotypic characterization

In the field evaluation trial, a total of 43 genotypes were evaluated in Random Block Design. The mpho-physiological data were recorded on 12 different yield attributing traits and were subjected to the analysis of variance (ANOVA).Standard error (S.E.) and critical difference (CD) were estimated using SPSSV19 software. The coefficient of correlation among all morpho-physiological traits at maturity was calculated manually using formulae in MS Excel. Based on similar coefficient, dendrogram was constructed using NTSYS-pc v2.1(Rohlf, 2000).

Molecular analysis

Young leaves were taken from 20 days-old plants of each genotype for DNA extraction. Genomic DNA was extracted by following the protocol described by Doyle and Doyle (1990) with required modifications. Purification was carried out to get RNA free DNA samples using RNaseA and quantification were carried out using 0.8% agarose gel. A total of 11 SSR primers (Table 2) were selected from published resources to express polymorphism among Indian mustard

genotypes. Polymerase chain reaction was performed in a total 10µl reaction volume in the Applied Biosystem Gradient Thermal Cycler. The composition of the reaction mixture was: 1.0 µl of (25 ng/µl) of DNA template, 1.0µl of 10x PCR buffer, 5 pmol of each primer, 0.2mM dNTP mix, 0.1 µl of *Taq* DNA polymerase, 0.5 µl of $MgCl_2$, 6.1 µl nuclease free water and final volume was made up the 10 µl. Polymerase chain reactions was carried out with following programme: one cycle of initial denaturation at 94 °C for 4 min; thirty-five cycles of denaturation at 94 °C of 30s, annealing for 30s, extension at 72°C for 45s min. The final extension of one cycle at 72 °C for 10 min was done before storage at 4°C. The amplified fragments were resolved on 3% agarose gel. Electrophoresed gels were documented under Gel Documentation System. Band sizes were determined by 100 bp DNA ladder. Bands were scored according to their size in base pair. Data was used to construct dendrogram using Power marker v3.25 (Liu and Muse, 2005). The analysis of major allelic frequency, polymorphism information content and genetic distance-based clustering were performed.

Results and Discussion

Morpho-physiological analysis

The analysis of variance for 12 yield and its attributing traits revealed presence of considerable amount of variation among different genotypes taken for present investigation (Table 2). The lowest and highest values for each character were recorded along with the mean performance which is also referred to as range. Plant height (cm), days to 50% flowering (DAS), days to maturity (DAS), main raceme length (cm), number (s) of primary branches/plant, number (s) of secondary branches/plant, siliqua length (cm), numbers of seeds/siliqua, number(s) of

siliqua/plant, 1000-seed weight (g), seed yield per plant (g) and harvest index (%) showed a wider range for trait values. The most important agronomic trait, seed yield per plant, showed a range between 5.0 g to 16.0 g (Table 3). The results observed were similar to the results reported by Vinu *et al.,* (2013) especially the most important seed yield per plant which showed a range between 5.90g to 15.59g. Results reported by Singh and Bhajan (2016) are also in accordance with the findings of present research. The study demonstrated that, variances due to replication were highly significant for plant height, main raceme length and number(s) of siliqua per plant; variances due to varieties were also found highly significant different for all the characters except number(s) of primary branches per plant, siliqua length, number(s) of seeds / siliqua and 1000-seed weight. Significant differences among the progenies/genotypes for all the traits investigated have been reported earlier (Singh and Bhajan, 2016).

Morpho-physiological data analysis divided the genotypes into two clusters *viz*., CI and CII. Cluster C-I divided into CI-A which further divided into CI-A(i) and CI-A(ii), cluster CI-A(i) consists of genotypes MRNJ-41, MRNJ-43, MRNJ-44, MRNJ-42, MRNJ-46, MRNJ-52, MRNJ-49, MRNJ-48, MRNJ-55, MRNJ-75, MRNJ-63, MRNJ-65, MRNJ-73, MRNJ-67, MRNJ-68, MRNJ-57, MRNJ-58, MRNJ-74, MRNJ-59, MRNJ-71, MRNJ-72, NRCHB-101and PM-30. It is also observed that NRCHB-101 shares close relationship with MRNJ-72 however, cluster CI-A (ii) consists of germplasm lines *i.e.*: MRNJ-45, MRNJ-47, MRNJ-53, MRNJ-61, MRNJ-62, MRNJ-70, MRNJ-64, MRNJ-80, MRNJ-60, MRNJ-78, MRNJ-56, MRNJ-77, MRNJ-79 and cultivated variety GM-2. Cluster CI-B consists of MRNJ-50 and MRNJ-51 (Fig.1, Table 4). In the dendrogram C-II is consisting of two cultivated varieties

namely: Rohini and JM-3 and a germplasm line: MRNJ-54 and all of three separated from the cluster C-I.

SSR marker analysis

In our study, a total of 65 alleles were identified. The genotype and allele number(s) per locus varied from 3-12. Maximum numbers of alleles were amplified with SR_9222 while minimum 3 with CN-52 (B-16) and SORF-73 (B-27) markers. The mean value for polymorphic markers was 5.99. The results of present study conducted showed somewhat resemblance with the study conducted by Qu *et al.,* (2012) using 217 genotypes and 37 markers, as the markers were referred from the similar research work. They observed 2 to 11 alleles at each locus with an average of 5.29 per marker whereas the polymorphism varied from 16.67% to 100%, however, they reported SR_9222 with 50% polymorphism and 6 numbers of alleles per locus it turned out to be the best marker in the present study may be due to the differences in germplasm lines taken, but surely the marker showed significant polymorphism and can be applied in crop improvement programmes in future.

Table.1 List of mustard germplasm lines and varieties with their collection source

S. No.	Germplasm lines	Source	S. No.	Germplasm lines	Source
1.	MRNJ-41	Directorate of Rapeseed-Mustard Research, Bharatpur	24.	MRNJ-64	Farmer's field of District Morena and adjoining areas
2.	MRNJ-42		25.	MRNJ-65	
3.	MRNJ-43		26.	MRNJ-67	
4.	MRNJ-44		27.	MRNJ-68	
5.	MRNJ-45		28.	MRNJ-70	
6.	MRNJ-46		29.	MRNJ-71	
7.	MRNJ-47		30.	MRNJ-72	
8.	MRNJ-48		31.	MRNJ-73	
9.	MRNJ-49		32.	MRNJ-74	
10.	MRNJ-50		33.	MRNJ-75	
11.	MRNJ-51		34.	MRNJ-76	
12.	MRNJ-52		35.	MRNJ-77	
13.	MRNJ-53	Farmer's field of District Morena and adjoining areas	36.	MRNJ-78	
14.	MRNJ-54		37.	MRNJ-79	Directorate of Rapeseed-Mustard Research, Bharatpur
15.	MRNJ-55		38.	MRNJ-80	
16.	MRNJ-56		*Varieties*		
17.	MRNJ-57		39.	Rohini	Kanpur , UP
18.	MRNJ-58		40.	JM-3	Morena
19.	MRNJ-59		41.	GM-2	Gujarat
20.	MRNJ-60		42.	NRCHB-101	DRMR, Bharatpur
21.	MRNJ-61		43.	PM-30	IARI, New Delhi
22.	MRNJ-62				
23.	MRNJ-63				

Table.2 Analysis of variance for yield and its attributes traits

Source of variation	DF	Plant height (cm)	Days to 50% flowering	Days to Maturity	Mean raceme length (cm)	Numbers of Primary branches/ plant	Numbers of secondary branches/ plant	Siliqua length (cm)	Numbers of seeds / siliqua	Numbers of Siliqua/ plant	1000-seed weight (g)	Seed yield/ plant (g)	Harvest index (%)
Replication	1	70.74**	0.19	0.57	13.44**	2.28	0.05	1.19	1.67	951.12**	0.49	0.04	0.03
Varieties	42	292.36**	57.67**	320.33**	120.38**	0.91	4.95*	0.34	3.55	2674.71**	1.29	11.19*	14.42**
Error	42	31.91**	2.26**	2.90**	4.82**	0.47	0.93	0.11	1.70*	79.04**	0.21	0.63	0.12

*, ** significant at 5 and 1% level of significance respectively

Table.3 Summary of Statistical analysis for morpho-physiological traits studied using SPSS V.19 software

Parameters/ Characters	PH	DF	DM	MRL	NPB	NSB	SL	NS	NSP	TGW	GY	HI
Mean	195.63	41.28	122.37	72.05	4.70	5.81	4.53	13.63	173.81	5.60	10.44	21.21
SE of Mean	1.84	0.82	1.93	1.18	0.10	0.24	0.06	0.20	5.58	0.12	0.36	0.41
SD	12.09	5.37	12.68	7.76	0.67	1.57	0.41	1.33	36.57	0.80	2.37	2.69
Variance	146.18	28.84	160.82	60.19	0.45	2.48	0.17	1.78	1337.36	0.65	5.60	7.21
Range	58	27	42	38	4	7	2	6	206	4	11	11
Minimum	162	29	99	57	4	4	4	11	109	4	5	16
Maximum	220	56	140	95	8	11	6	17	314	8	16	27

PH-plant height, DF - days to 50% flowering, DM-days to maturity, MRL-main raceme length, NPB- number of primary branches/plant, NSB- number of secondary braches/plant,
SL-Siliqua length, NS- number of Seeds /Siliqua, NSP- number of Siliqua per plant, TSW-1000-seed weight, GY- seed yield, HI- harvesting index, SE-Standard Error, SD-Standard Deviation

Table.4 Polymorphic Information Content and Statistical Details of SSR markers

| Marker	Forward sequence 5'-3'	Reverse sequence 5'-3'	Major Allele Frequency	Allele Number	Gene Diversity (%)	PIC
SR_9222	CACCGAACAAAA CTGAGGGT	CGTTTCACTGCGT TCTACCA	0.1628	12	88.75	0.8768
BRMS_324	AACTTAACCGAA ACCGAGSTAGG	AATCTCGAAATT CATCGACTTCC	0.2093	7	83.72	0.8160
SR_7223	AGGACCCGACTT TCCTTGTT	ACCAAACTCGGC GTACAAAT	0.3023	6	78.42	0.7517
SSR_Na10D09	AAGAACGTCAAG ATCCTCTGC	ACCACCACGGTA GTAGAGCG	0.4884	5	68.69	0.6475
BRMS-240	CAAGAGTATTTG TGTGGGTTGAC	AAATAACGAACG GAGAGAGAGG	0.3721	5	75.39	0.7165
SSR_Na10-D11	GAGACATAGATG AGTGAATCTGGC	CATTAGTTGTGG ACGGTCGG	0.4419	6	68.36	0.6330
SR_94102	ATCCCCAAACTA CCCTCACC	AGGATGAGCAAA GGAAAGCA	0.4419	4	67.82	0.6219
BRMS-093	TCCAAGTAGACC GAATCAAGAGT	ATAAATCGAACC TGAAACCATGT	0.3953	6	73.88	0.6986
CB-10065	CGGCAATAATGG ACCACT	CGGCTTTCACGC AGACTTCG	0.2093	8	84.91	0.8311
CN -52 (B-16)	CCGGCTTGGTTC GATACTT	TTGCGAATCTTTA AGGGACG	0.1934	3	58.29	0.3219
SORF-73 (B-27)	CGTGGGCCAAGC TTAGATTA	CGTTCAAGAAGA CACAGATCAAA	0.1835	3	55.32	0.2957
Mean			**0.3091**	**5.99**	**73.05**	**0.6555**
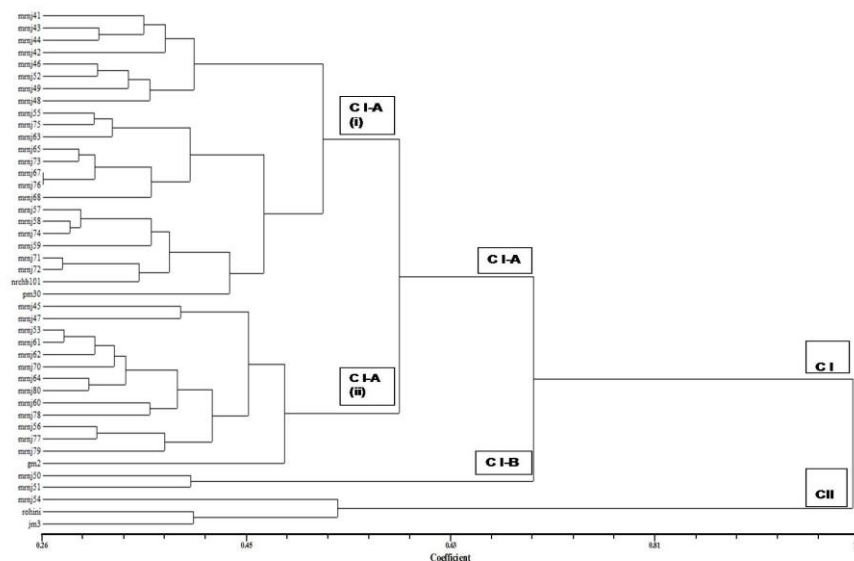
Fig. 1 Dendrogram of pooled morphological data using NTSYS pc v2.1

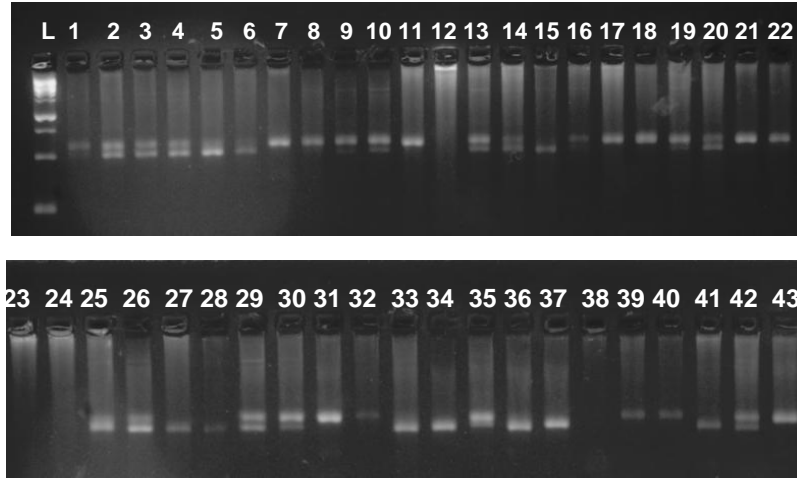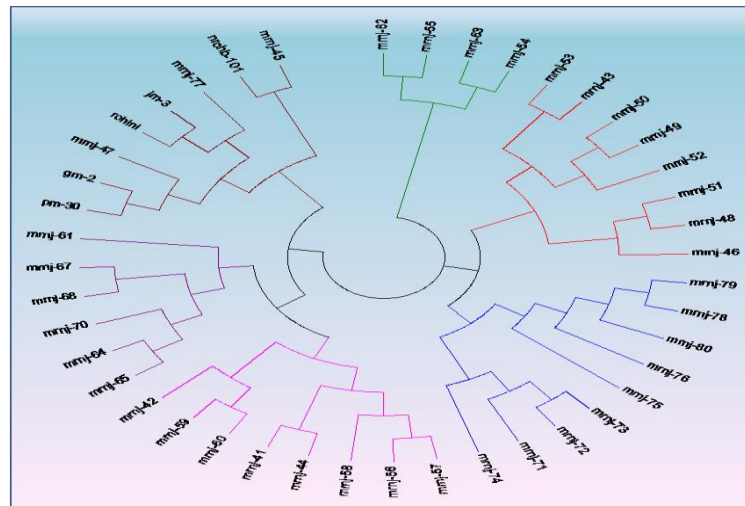**Fig.2** Gel image representing PCR product of SR_94102 with 43 mustard genotypes

Fig.3 Dendrogram of mustard genotypes based on SSR data using Power marker software

Microsatellites were used for DNA based diversity investigation among mustard genotypes successfully amplified with template DNA of studied genotypes. The multiple banding patterns (Fig. 2) were found with SSR markers in mustard cultivars. Similar reports are also available regarding appearance of multiple bands as the result of amplification of more than one locus by each SSR (Holton *et al.*, 2002). This may be due to availability of multiple primer binding sites (Winter *et al.*, 1999).

In our study, PIC value of the markers varied from minimum 0.2957 with primer SORF-73 (B-27) to a maximum 0.8768 with SR_9222 with an average of 0.6555. The average PIC values were comparable as reported (Patel *et al.*, 2018) where the PIC value ranged from 0.427-0.730 with an average of 0.555 and Shyam *et al.*, (2020) who reported polymorphism information content (PIC) value of the markers varied from minimum 0.51for SSR Na10-D07 to maximum 0.73 with primer BRMS-098 with a mean value of

0.62 (Table 5). PIC values reported by Bharti *et al.,* (2018) ranged from 0.34 to 0.49 with an average of 0.41 while Gupta *et al.,* (2014) reported low PIC value 0.281 for SSR markers during their study on molecular diversity analysis of mustard. Lower PIC values may be attributed either due to less informative SSR markers, or slighter diversity amongst the tested genotypes. The mean PIC values ranged 0.38 to 0.96 were observed by Avatar *et al.,* (2016), where the utmost value of PIC obtained was to some extent higher than our study. In our study, the selected 11 SSR markers showed polymorphism they showed sufficiently high sensitivity to detect DNA polymorphisms among the 43 *B. juncea* germplasm lines and cultivars. The results obtained in the present study extended useful information. SSR markers are informative which could be utilized for characterization of germplasm lines. SSR has been effectively utilized as efficient tool for genetic characterization of many plant species including oilseed rape (Shyam *et al.,* 2020).

The genetic relationships among mustard genotypes are presented in SSR based UPGMA tree. Major three clusters were obtained *i.e.*, C-I, C-II and C-III, in which cluster C-I consisted of germplasm lines MRNJ-62 and MRNJ-65 clustered together and MRNJ-63 and MRNJ- 54 in other. Cluster C-II was again divided into sub clusters 'C-II(a)' and 'C-II(b)', cluster C-II(a) consisted of MRNJ-53, MRNJ-43, MRNJ-50, MRNJ-49 and MRNJ-52 in one group and MRNJ-51, MRNJ-48 and MRNJ-46 in another, whereas C-II(b) consisted of MRNJ-79, MRNJ-78, MRNJ-80, MRNJ-76 and MRNJ-75 in one group and MRNJ-73, MRNJ-72, MRNJ-71, MRNJ-74 in another group. Cluster C-III was divided into 2 subgroups C-III(a) consisting of MRNJ-57, MRNJ-56, MRNJ-58, MRNJ-44 and MRNJ-41 in C-III a(i)I and MRNJ-60, MRNJ-59 and MRNJ-42 in C-IIIa(i)II. Germplasm lines

MRNJ-65, MRNJ-64, MRNJ-70, MRNJ-68 and MRNJ-67 fallen in C-IIIa(ii) I cluster and MRNJ-61 in C-III a(ii)II whereas C-IIIb consists of C-IIIb(i) in which PM-30, GM-2 and MRNJ-47in one group CIII b(i) I and Rohini, JM-3, MRNJ-77 in another naming C-IIIb(ii)II. Other subgroup of the cluster C-III(b), *i.e.* C-IIIb (ii) consists of NRCHB-101 and MRNJ-45 (Fig 3). Earlier Qu *et al.,* (2012) has also reported similar clustering after using SSR markers; the clustering was according to the known pedigrees. In another study Vinu *et al.,* (2013) worked on assessment of genetic variability among Indian mustard genotypes using 134 microsatellites and obtained four major clusters. Different reports on clustering of mustard genotypes after application of microsatellites are available (Chen *et al.,* 2011; Baghel *et al.,* 2020). Clustering of genotypes shows a very diverse pattern according to genotypes and SSR markers.

It was clearly revealed from dendrogram that germplasm line MRNJ-54 falling with varieties Rohini and JM-3 and MRNJ-71 and MRNJ-72 with NRCHB-101 together there is possibility of existing many traits to be similar whereas the germplasm lines MRNJ-50 and MRNJ-51 falling extremely apart from whole of the cluster shows the possibility of discovering new traits in them which could be further assisted in conventional and molecular breeding programmes. The dendrogram based on SSR data showed significant diversity; it grouped the genotypes into three clusters. The clusters were further divided into sub clusters revealing close relationships of MRNJ-47 with varieties PM-30 and GM-2 having the possibilities of similarity in many of the traits whereas MRNJ-77 showed close relationship with varieties Rohini and JM-3. MRNJ-45 shared close resemblance with NRCHB-101.Germplasm lines MRNJ-62, MRNJ-65, MRNJ-63 and MRNJ-54 formed a separate cluster in dendrogram revealing entirely

different genetic traits whereas through morpho-physiological cluster diagram, it was revealed that germplasm lines MRNJ-50 and MRNJ-51 are falling whole apart from the entire cluster indicating possibility of entirely different morpho-physiological traits. These unique germplasm lines could surely assist to give major support in the future molecular and conventional breeding programmes.

In conclusion the molecular profiling has been the preferred choice of breeding as these are more reliable and authentic and less influenced by environmental fluctuations. Therefore, SSR marker data described in this work would surely provide a useful starting point for further diversity analysis in future programmes aiming in mustard crop improvement at molecular level.

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