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RESEARCH ARTICLE

Evaluation of genetic diversity among Nepalese rapeseed germplasm accessions using SSR markers

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Abstract

Rapeseed is an important oilseed crop in Nepal which covers significant position among cash crops in terms of area and production. We have used already published SSR markers to study the genetic diversity among the rapeseed germplasm accessions. Altogether 20 SSR primers were tested for 48 Nepalese rapeseed germplasms. Out of 48 SSR markers, 11 markers showed polymorphism. Polymorphism Information Content (PIC) value ranged from 0.40 to 0.84. Out of eleven polymorphic markers, NA10-C01a showed the highest level of polymorphism (PIC=8.4). Percent of polymorphic marker was 55% where alleles per polymorphic loci were 2.36 and the sum of total polymorphic alleles was 26. The cluster diagram categorized studied germplasm and cultivars in two major groups with maximum similarity of 36.05%. Hundred percent similarities were observed between genotypes collected from Ramechhap (Rato Tori) region with accession number NGCRO2790 and Sindhupalchok (Sano Tori). We also noted 100 % similarity between the two released varieties, namely Sumle Tori 3 and Bikas, whereas more than 90% similarity was observed between the three released varieties Unnati, Sumle Tori and Bikas. The Principle Component Analysis (PCA) also support the findings obtained by cluster analysis, where all the genotypes also characterized in two major groups. We conclude that greater diversity is present among the studied accessions and SSR markers are very handy and applicable tool for the diversity assessment of rapeseed germplasm. Therefore we can exploit the available rapeseed diversity of the country for the development of better rapeseed varieties to cope with biotic and abiotic stresses and climate change issues.

Key words: Diversity, rapeseed, SSR marker, polymorphism information content

Introduction

Oilseed crops cover significant position among cash crops in terms of area and production in Nepal. Oilseed crops cover about 5% of total cropped area and ranked in the sixth position after cereal crops and legumes in terms of acreage while for oilseed crops, the average area, production and yield were 224582ha, 194536 ton and 866kg/ha respectively during 2013/2014 (MoAD, 2014). Oils and fats are very good source of calories (Chaudhari, 2008). Rapeseed and mustard are the major oilseed crops cultivated in all agro-ecological regions of the country from high hill (2500 masl) to terai and inner terai (58 masl) (Joshi *et al.*, 2017). Rapeseed and mustard cover about 80% of total oilseed crop area which is 191792 ha (MoAD, 2014). Earlier 92 rapeseed accessions were characterized based on morphological trait and evaluated at National Agriculture Genetic Resource Center, Khumaltar (Joshi *et al.*, 2013).

At present 354 rapeseed mustard, 77 groundnut, 67 niger, 43 linseed and 34 sesame accessions were maintained and evaluated at National Oil Research Program (NORP), Nawalpur, Sarlahi, Nepal (NoRP, 2016). The rapeseed and mustard seed contains 40-45% oil and 24% protein (NoRP, 2016). Rapeseed and mustard oil is nutritionally far superior to any other vegetable oils, because of sufficiently low level of saturated fatty acids (7%), moderate level of poly unsaturated fatty acids, linoleic (omega-6) and linolenic (Omega-3) which is highly balance (12:10) and higher amount of monounsaturated fatty acids like oleic and erucic acid (70%) (NoRP, 2016). Rapeseed mustard oil is safe for healthy people as well as for people with weak heart and those suffering from other chronic diseases. For the diversity assessment of rapeseed, the SSR markers have been used extensively all over the world viz. Asia, Africa, Europe and Australia. The reason behind the extensive use of SSR markers for the diversity assessment is the co-dominant nature of the marker. Due to this, they determine the heterozygosity of the genotype by the presence of polymorphic parental alleles which facilitates the testing of purelines. Similarly SSR markers are highly informative, highly polymorphic and multi-allelic, that are experimentally reproducible and transferable among the wild relative species (Mason 2015). The other relevant applications of SSR markers are to determination of genetic distance, to estimate the gene flow and crossing over rates and for the construction of dendrogram and phylogenetic tree, (iv) for the QTL analysis and for marker assisted selection (MAS) (Jonah *et al.*, 2011; Kalia *et al.*, 2011).

Very limited reports are available from Napale for the systematic characterization of the rapeseed germplasm at morphological level. For rapeseed germplasm till date from Napale there were no published reports on the utilization of the SSR markers for the diversity study. Hence, the present study was aimed to analyze the molecular diversity of Nepalese rapeseed accessions obtained from various districts from east to west for which no morphological or genetic characterization has been done. This study will help the breeders to pick up the most divergent germplasm for the development of breeding material in rapeseed.

Materials and methods

Seeds of 48 germplasm accessions of rapeseed were obtained from National Oilseed Research Programme, Nawalpur Sarlahi and from National Gene Bank, NARC, Khumaltar. The obtained seeds were collected from different districts of Nepal by National Gene Bank. Total 20 SSR markers were chosen for the study. Altogether 18 primers were selected from Pratap *et al.,* (2015) and two primers from Abbas *et al.,* (2009). The seeds were allowed to germinate in the greenhouse condition in a tray for 10 days. Once the seeds fully germinated after 10 days of sowing, 5g leaves of each genotype were obtained for the DNA extraction. For the extraction of the DNA modified CTAB method was used as suggested by Wang *et al.,* (2009).

PCR amplification

PCR master mix 2X (Go Taq Green) consists of Taq DNA pol, reaction buffer ph 8.5, MgCl2 (3mM), 400µM of dATP, dTTP, dGTP and dCTP respectively. Before PCR amplification process, DNA stock solution was converted into the working solution via dilution. 10% DNA solution was prepared as working solution and finally PCR cocktail was prepared. Forward primer 1.5µl, reverse primer 1.5µl, Go Taq Green 7.5 µl, nuclease free water 2.5µl, working DNA solution 2µl was added for final volume of 15µl of PCR cocktail per sample. PCR master mix 2X (Go Taq Green) consists of Taq DNA pol, reaction buffer ph 8.5, MgCl2 (3mM), 400µM of dATP, dTTP, dGTP and dCTP respectively.

The first amplification cycle consisted of initial denaturation at 94°C for 5 min followed by 45 cycles each of 30 sec at 94°C for denaturation, primer annealing at 55°C for 30 sec, primer extension at 72°C for 45 sec, and a final extension step at 72°C for 7 min. Similarly amplification cycle consisted of initial denaturation at 95°C for 1 min followed by 35 cycles each of 1m at 94°C for denaturation, primer annealing at 35°C for 1m, primer extension at 72°C for 1min, and a final extension step at 72°C for 7 min. After the completion of PCR amplification, the samples were kept at -20°C until gel electrophoresis.

Statistical analysis

In particular it allows identifying the principal direction in which the data varies. The first component extracted in a principal component analysis accounts for a maximal amount of total variance in the observed variables. Under typical condition this means, that the first components correlates with at least one of the observed variables. The second component extracted has two important characteristics. First this component accounts for a maximal amount of variance in the data set that was not accounted for by the first component and second it is uncorrelated with the first component. The remaining components extracted in the analysis display the same two characteristics that each component accounts for a maximal amount of variance in the observed variables that was not accounted for by the proceeding components and is uncorrelated with all of the preceding components (Coghlan, 2013). Here in our study cluster analysis and dendrogram construction was done through statistical software Minitab 17. MPJ. The profile data produced by SSR markers were scored manually for each fragment in every accession for each primer pair and recorded as 1 representing the presence of a fragment and 0 representing the absence of a fragment. The following formula was used to calculate the polymorphic information content

$$(\text{PIC}) \text{ per marker} = 1 - \sum_{J=1}^{n} Pij^2$$

Where, *Pij* is the frequency of the j^{th} allele of the i^{th} marker locus and *n* is the total number of alleles.

Results and discussion

PIC values are important criteria to access diversity or level of polymorphism. The PIC value will be almost zero if there is no allelic variation and it can reach a maximum of 1.0 if a genotype has only new allele which is a rare phenomenon (Thatikunta *et al.*, 2016). All-together 20 SSR primers were tested for 48 Nepalese rapeseed germplasm and cultivars. Out of them 11 markers showed polymorphism with PIC value ranging from 0.40 to 0.84. Four primers were null and five of them were monomorphic. Total tested primers showed wide range of base pairs ranging from less than 100 to 750bp. Polymorphic markers such as BN25A, NI03-H07a, RA02-A04a showed lesser number of base pairs (<100). Similarly primers BRMS-017, BRMS-030, BrgMS1237, BrgMS70, MB5, BRMS-003, NI-F02a showed base pairs ranging from 100 to 250. Likewise primers BrgMS329, BrgMS339, BrgMS710, BRMS-033, NA10-C01a showed base pair range greater than 250 bp. The greatest base pair range (120-750 bp) was exhibited by the primer NA10-C01a. For all polymorphic primers except NA10-C01a, the number of polymorphic alleles was two. But for the primer NA10-C01a that was six, also the highest PIC value was observed for the same primer (0.84). The sum total of polymorphic alleles was 26 whereas the total number of alleles obtained was 746.

Chung *et al.*, (2006) had an excellent results based on different *Brassica juncea* breeding lines and cultivars. Among 194 SSR primers tested, average polymorphic alleles per locus were found to be 3.46 while 194 polymorphic alleles were demonstrated for 77 genotypes which is significantly outstanding than our finding. On the other hand the polymorphic bands ranged from 1 to 11 which is very much higher than that of our finding. Having all these outstanding results the PIC value between these two researches are quite similar. The PIC value ranged within 0.1 to 0.8 which is nearly equal 0.84 as per our findings. Likewise maximum percent of polymorphic primers was 100% which is quite outstanding. The huge difference in the results can be due to better selection of primers and genotypes by the previous researchers.

Mason (2015) reported the very high level of polymorphism in different Indian B. *juncea* cultivars and varieties where 109 bands were obtained from 10 ISSR markers of which 78 were polymorphic. The percent polymorphism was higher (71.56 %) as compared to 55% for our work. Similarly the PIC value was quite excellent (0.75-0.99). They also reported that alleles per polymorphic loci to be 7.8 which were very much higher than that of 2.36. The better results obtained by Mason (2015) can be credited to the better primer selection and selection of advanced breeding lines for the study.

Table 1: Diversity parameter among the rapeseed varieties

Diversity parameters	Number
Total number of sample analyzed	48
Total SSR marker tested	20
SSR markers not amplified	4
Total number of SSR markers amplified	16
Total polymorphic markers	11
Total monomorphic markers	5
% of polymorphic markers	55%
Total number of polymorphic bands	746
Total number of polymorphic alleles	26
Alleles per polymorphic loci (total numbers of polymorphic alleles/total number of polymorphic markers)	2.36
Average PIC value	0.5

Cluster analysis

The UPGMA cluster diagram of 48 rapeseed germplasms resulted in major groups A and B and the maximum % of genetic diversity between the germplasm accessions under the two major groups was 64% (Figure not shown). These two clusters were further divided into A1 A2 and B1 B2. A1 was further divided into A1.1 and A1.2 . Again A1.1 was divided into A1.1.1 and A1.1.2 again A1.2 was divided into A1.2.1 and A1.2.2. B2 was further divided into B2.1 B2.2. B21 was further categorized into B2.1.1 and B2.1.2. Cluster A consisted germplasms with 23 accessions. Cluster B consisted with 25 accession numbers. Maximum 36% similarity was observed between all 48 genotypes and at the same time 100% similarity was observed between the varieties Sumle Tori and Bikas and between NGRCO 2790 and NGRCO 2797. District wise genotype categorization and their similarity among other is presented in table 2.

The significant differences were observed due to the selection of the diverse genotypes. Due to the co-dominant nature of the SSR maker, homozygote alleles can be easily distinguished from heterozygote alleles. Primers NI-F02a, RA02-A04a and BrgMS339 (Gel picture not shown) showed the heterozygous bands, which confirm the better diversity among the genotypes. These findings are in accordance with the Pratap *et al.,* (2015).

Polymorphic information content (PIC) and nature of the primers

The polymorphic information content ranged from 0.40 to 0.84 in our study (Table3). Four primers didn't work in our study. For co-dominant markers like SSR, the expected range of PIC value lies within the range of 0.5 to 0.8. Out of 20 primers studied only 11 primers showed consistent banding patterns which generated 26 polymorphic bands with an average of 2.36 alleles per polymorphic loci i.e. 55% primers were being polymorphic. Primer NA10-C01a produced the highest number of polymorphic bands which was six remaining all primers gave only two polymorphic bands. Here some primers were unable to amplify for some genotypes, although the same primer produced polymorphic bands for other genotypes. For primer NA10-C01a only 40 germplasms were amplified out of 48 Germplasm accession and cultivars, while eight germplasms remained unamplified. This is possible due to sequence non complementarity of the primer to the DNA of respective germplasm. The sum total of polymorphic alleles was 26 whereas the total number of alleles obtained was 746. Only 11 primers out of 20 were seen polymorphic while all primers were polymorphic in case of Pratap *et al.,* (2015). The reason behind this could be the selection of the different material selected in both the studies.

Principle component analysis

In our study, the first principle component explained 22% of the total variation among the genotypes while 2nd one accounted for 13.5%. The highest Eigen value was 6.1 from the principle component 1, while principle component 2 gave 3.8. PCA partitioned total variance into 10 principle components contributing maximum to the total diversity among the genotypes. The UPGMA cluster analysis also supports the PCA results. Those genotypes which are grouped together in UPGMA cluster analysis are also grouped together in PC analysis. Considerable diversity has been observed between the germplasms collected from same geographical location. This unpredictable genetic dissimilarity might have occoured due to two major factors. It might be possible due to plant introduction from different geographical location in the past but in long run the germplasm was thought to be native to the particular district or region. Secondly Mahabharat range of Nepal is considered as one of the secondary centers of origin for rapeseed (Joshi *et al.,* 2017), due to which huge diversity might have occurred within the narrow geographical location, due to the cross pollination and natural breeding. The other reason behind huge diversity in Nepalese rapeseed germplasm accession and greater diversity of the crop in India and the exchange of the genotypes cannot be denied due to open borders of the country. Similar study carried out by Singh *et al.,* 2013 to study diversity among 44 genotypes of *B. juncea*. The principal component analysis and structure analysis exhibited similar results to the cluster analysis (Chung *et al.,* 2006) where 77 rapeseed accessions (*Brassica napus*), including 22 varieties and 55 advanced breeding lines were analyzed by 47 sequence-related amplified polymorphism (SRAP) and 56 simple sequence repeat (SSR) primers.

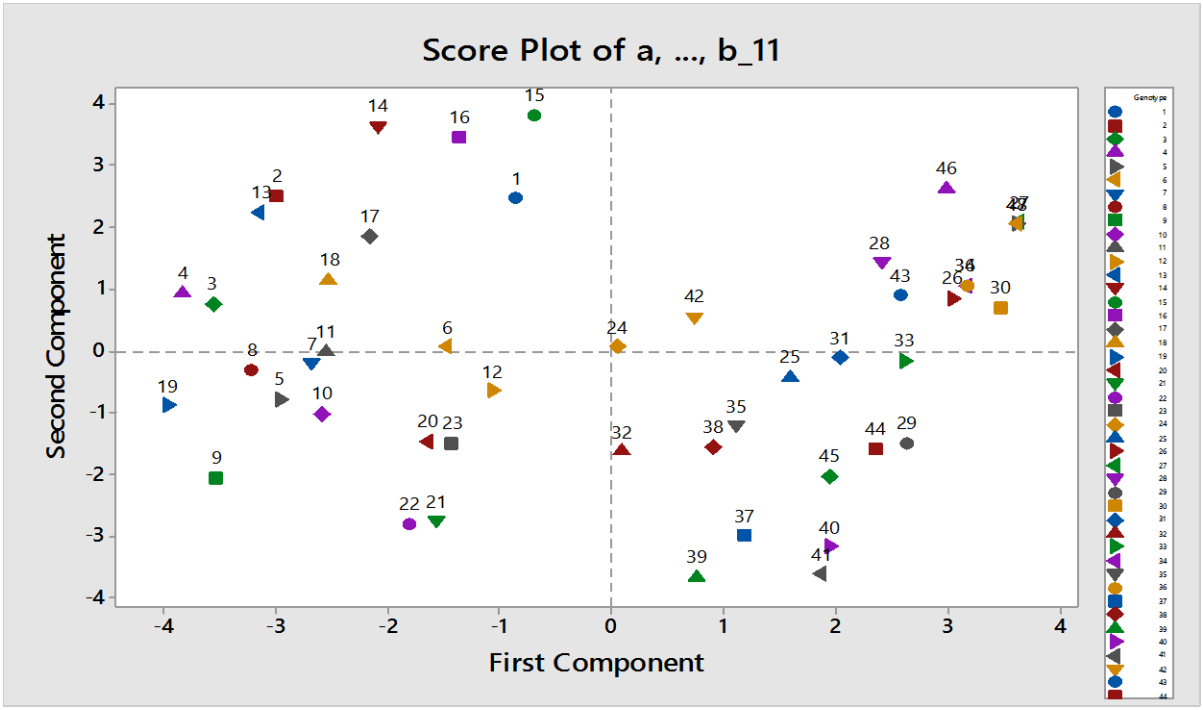
Table 2: District wise genotype categorization and their similarity among each other

Sr. No.	District	Name of the genotype similarity pattern and	Remark
1	Sunsari	NGCRO 2746 and NGCRO 2747 (65%)	-Nil-
2.	Kavre	NGCRO 2749, NGCRO 2752, NGCRO 2753, and NGCRO 2761 (50%)	All these germplasm accession possessed greater amount of variability although they belong to the same location
3	Nuwakot	NGCRO 2750 (50%)	Only one germplasm was observed to be 75% similar with NGCRO 2756, obtained from Dolakha.
4	Rasuwa	NGCRO2751,NGCRO2762,NGCRO2769, NGCRO2777, NGCRO2778, NGCRO2781 and NGCRO 2783 (50%)	Due to the highest number of germplasms from same location, greater variability was observed between them
5	Dolakha	NGCRO2754, NGCRO2756, NGCRO2757, NGCRO2760, NGCRO 2766 and NGCRO 2767 (Variable)	NGCRO 2754 and NGCRO2756 were 65% similar likewise germplasm NGCRO 2757 and NGCRO 2767 were almost 80% similar to each other. Interestingly the germplasms NGCRO 2754, NGCRO 2757, NGCRO 2767 and NGCRO 2756 were just 50% similar to NGCRO 2766, while other germplasms obtained were 45% similar to NGCRO 2760. This looks peculiar since all the accessions were obtained from same district.
6	Saptari	NGCRO 2758, NGCRO 2764, NGCRO 2773 (Variable)	Germplasm accession NGCRO 2758 and NGCRO 2764 were just 50% similar to each other, while the remaining germplasm NGCRO 2773 was very much diverse from the earlier two germplasm having only 36% similarity.
7	Salyan	(70)	-Nil-
8	Sindhupalc-howk	NGCRO 2763 and NGCRO 2765 (Variable)	Two Germplasm accession NGCRO 2768 and 2797 were 36% similar to each other, which was the maximum similarity for any genotype, on the other hand NGCRO 2797 and 2807 were comparatively closer to each other having about 50% similarity.
9	Kailali	NGCRO 2768 NGCRO 2797 and NGCRO 2807 (36%)	-Nil-
10	Ramechhap	NGCRO 2771, NGCRO 2785 and NGCRO 2790 (Variable)	Germplasm accession NGCRO 2771 and NGCRO 2785 were 36% similar to each other, while NGCRO 2785 and NGCRO 2790 were 50% similar to each other.
11	Banke	NGCRO 2787 (90 %)	-Nil-
12	Baitadi	NGCRO 2795 (80%)	Similar to NGCRO 2785 obtained from Ramechhap
13	Tanahun	NGCRO 2798 (90%)	Similar to NGCRO 281 obtained from Chitwan
14	Parbat	NGRCO 2801 (65%)	Similar to a released variety Bal tori
15	Chitwan	NGCRO 2779 and NGCRO 2814 (50%)	-Nil-
16	Morang	Morang tori (80%)	Similar to NGCRO 2807 obtained from Sindhupalchok
17	Surkhet	Surkhet Tori (80%)	Similar with a released variety Preeti
18	-	Unnati (80%)	Similar with Sumle tori and Bikas, while Sumle Tori and Bikas were almost 100% similar

Table 3: Demonstrating the results regarding primer code and PIC value along with the total number of polymorphic

Sr No	Primer code	Molecualr weight range (bp)	Amplified sample number	No. of polymorphic alleles	PIC value
1	NA10-C01c	-	-	-	-
2	BRMS-017	180-200	48	2	0.50
3	BRMS-030	200-210	48	2	0.50
4	BN6A3	-	-	-	-
5	NA10-C01a	120-750	40	6	0.84
6	NI02-D08a	120	45	-	-
7	BrgMS329	370-380	42	-	-
8	BrgMS1237	210-230	47	2	0.40
9	BN25A	100	48	-	-
10	BrgMS339	250-300	43	2	0.45
11	NI03-H07a	100	48	-	-
12	BrgMS70	180-220	48	2	0.41
13	MB5	95-105	48	-	-
14	MR52a	-	-	-	-
15	OL10-A03a	-	-	-	-
16	RA02-A04a	< 100	48	2	0.50
17	BrgMS710	320-350	41	2	0.50
18	BRMS-033	230-260	48	2	0.48
19	BRMS-003	100-150	48	2	0.50
20	NI-F02a	170-200	56	2	0.50

Fig1: The score plot of first two components of rapeseed germplasms and cultivars



Conclusion

The polymorphism observed between the studied germplasm accessions reflects the presence of differ alleles and diverse nature of the germplasm. On the other hand SSR markers were the best applicable tool for the diversity analysis. These SSR markers were able to detect even the meager amount of polymorphism between the studied varieties. Therefore we can conclude that SSR markers are very handy tool for the analysis of biodiversity in different crops available in the country. The study revealed that there is a greater biodiversity among the studied rapeseed germplasms and cultivars available in the country. Rapeseeds were not characterized at molecular level in the past; therefore there is a better scope and space for the breeders for further investigation and the strengthen the research in the field of oilseed crop by synchronizing the morphological diversity with the genetic diversity, with the aim of development of better rapeseed varieties by the exploitation of naturally available diversity in the country. The diversity can be very fruitful for designing the breeding programmes to cope with biotic and abiotic stresses and climate change issues.

References

1. Ayub Khan, M., Ashiq Rabbani, M., Munir, M., Khan Ajmal S., and Azim Malik, M. 2008. Assessment of genetic variation within Indian mustard (*Brassica juncea*) Germplasm using random amplified polymorphic DNA Markers. J. Integrative Plant Bio., 50(8): 385-392.
2. Chaudhary, R. N. 2008. Winter oilseed crops report. In: Proc. of 27th National Winter Crops (Oilseeds) Workshop, Nepal Agricultural Research Council, National Oilseeds Research Programme, Nawalpur, Sarlahi, Nepal, pp.1-10.
3. Chung, A.M., Staub, J. E. and Chen, J. F. 2006. Molecular phylogeny of cucumis species as revealed by consensus chloroplast SSR marker length and sequence variation. Genome 49: 219–229
4. FAO, World Food and Agriculture. Statistical Yearbook. 2013. pp. 289. Available online at www.fao.org
5. Coghlan, A. 2013. A Line Book of R for Multivariate Analysis, pp. 5.
6. Ghimire, T. B. 2000. Sunflower production technology (In Nepali). National Oilseed Research Program, Nawalpur, Sarlahi, pp.50.
7. Hasan, M., Seyis, F., Badani, A., Pons-Kuhnemann, J., Friedt, W., Luhs, W. and Snowdon, R. 2006. Analysis of genetic diversity in the *Brassica napus* L. gene pool using SSR markers. Genet Resour Crop evn., 53: 793-802.
8. Jonah, P., Bello, L., Lucky, O, Midau A, and Moruppa S. 2011. Review: The importance of molecular markers in plant breeding programmes. Global J. Sci. Front Res., 11(5):1-11.
9. Joshi, B. K., Ghimire, K. H. and Yadav, N. K.. 2013. Agricultural plant genetic resources management and agriculture in Nepal. AFACI 1st International Training Workshop on Germplasm Management System (GMS) in Asia, Suwon, Korea. pp.139-177.
10. Joshi, B. K. and Acharya, A. K. 2017. Conservation and utilization of agricultural plant genetic resources in Nepal. Proceedings of 2nd National Workshop, 22-23 May 2017, Dhulikhel; NAGRC, FDD, DoA and MoAD; Kathmandu, Nepal.
11. Kalia, R. K., Rai, M. K., Kalia, S., Singh, R., Dhawan, A. K.. 2011. Microsatellite markers: An overview of the recent progress in plants. Euphytica. 177:309–334.
12. Mason, A. S. 2015. SSR genotyping. In: Batley J. editor. Plant Genotyping. Springer; New York, NY. pp. 77–89.

13. MoAD. 2014. Statistical information of Nepalese agriculture. Government of Nepal, Ministry of Agricultural Development. Agri-business Promotion and Statistics Division, Singhadurbar, Kathmandu, Nepal.
14. NoRP. 2016. Annual report 2072/73(2015/16). Oilseeds Research Program, NARC, Nawalpur, Sarlahi, Nepal.
15. Pratap, P., Thakur, A.K., Meena, P.D., Meena, H.S., Sharma P., Singh D., and Mujumdar, P. 2015. Genetic diversity assessment in Indian mustard (*Brassica juncea* L.) for Alternaria blight tolerance using SSR markers. *J.* Oilseed Brassica. 6(1): 175-182.
16. Thatikunta, R., Sankar, A.S, Sreelakshmi, J., Palle, G., Leela, C., Rani C.V.D. , Gouri Shankar, V., Lavanya, B., Narayana Reddy, P. and Dudhe, M.Y. 2016. Utilization of *in silico* EST-SSR markers for diversity studies in castor (*Ricinus Communis* L.). Physiol. Mol. Biol. Plants 22(4):535–545.
17. Wang, M. L., Barkley, N. A. and Jenkins, T. M. 2009. Microsatellite markers in plants and insects Part I. Applications of biotechnology. G3-Genes Genom Genet 3:54–67.