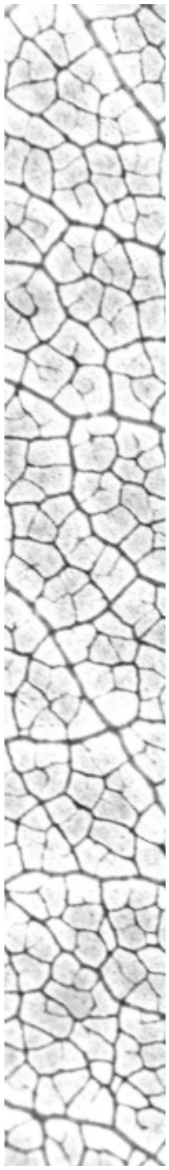

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Assessing genetic diversity for drought and heat stress tolerance of Nepalese wheat genotypes by SSR markers

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Abstract
The productivity of wheat in Nepal is low compared to the world average and very low compared to countries like New Zealand and Ireland, and it has remained nearly stagnant in the past ten years. Genetic factors, water and climate seem to be a limiting factor for wheat productivity in Nepal. Breeding for tolerance to biotic and abiotic stress and study of their genetic stability under stress are important for improving the productivity of wheat. We assessed the genetic diversity of 20 genotypes of wheat released or in the pipeline in Nepal, by 12 SSR markers linked to drought tolerance and 4 SSR markers linked to heat stress tolerance. The first set clustered 20 genotypes into 4 clusters, 3 of which further sub clustered into 8 sub clusters. 2 of the genotypes, namely BL-4707 and NL-1325, are distantly related to the rest of the genotypes. The second set of SSR markers clustered 20 genotypes into 5 clusters, 2 of which further subclustered into 4 subclusters. 2 of the genotypes, namely NL-1247 and NL-1325, are distantly related to each other and the rest of the genotypes. This study identified both closely related genotypes and distantly related genotypes of wheat in alleles presumably linked to drought and heat stress tolerance. The finding of this study is expected to be useful for breeding for drought and heat stress tolerance and study of the interaction of genotypes and environment as well.

Keywords: heat stress, drought, SSR, wheat, breeding, productivity

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INTRODUCTION

Wheat (*Triticum aestivum*) is the largest food crop to cover the earth's surface (218.54 million hectares in 2017) and the second largest crop after maize in terms of the production (771.71 million tons in 2017) in the world (FAO 2018). Wheat is the largest deficit item in the developing country food basket. Between 1970 and 2010, more than half of the increment in wheat consumption was met by increased wheat imports, and several countries became totally dependent on imports for wheat (FAO 2013). The Cereal Import Dependency Ratio, an indicator of a country's dependency on the import of cereals calculated as the average of three years by FAO, of Nepal was 1.2 in 1990. It has risen to 1.7% in 2000, 3.9% in 2014 and 7.6% in 2016 (FAO 2015, 2018). Nepal imported 0.19 million metric ton of wheat worth NRs. 5.2 billion (approx. 48 million USD) in 2016/17 (MoALMC 2018). The limiting factors among abiotic stresses for wheat production worldwide are drought and heat (Prasad *et al.* 2011, Liu *et al.* 2016, Lesk *et al.* 2016).

Drought and heat tolerance are major global strategies in wheat breeding (Lamaoui *et al.* 2018, Senapati *et al.* 2019, Tilman *et al.* 2011, Tricker *et al.* 2018). Information on genetic diversity, relatedness and distance is a foundation for plant breeding for desired traits and DNA markers for alleles and traits are valuable tools for it (Fleury *et al.* 2010, Henkar *et al.* 2016, Longin *et al.* 2015, Vagndorf *et al.* 2018). SSRs are preferred markers for genetic diversity, linkage mapping, association studies, and marker-assisted selection due to its high reproducibility, multi-allelic nature, co-dominant inheritance, robust amplification (Jaiswal *et al.* 2017, Röder *et al.* 1998, Sajjad *et al.* 2018) and have been extensively used for the assessment of genetic diversity and molecular genetic mapping of wheat (Abbasov 2018, Henkrar *et al.* 2016, Song *et al.* 2005, Tian *et al.* 2015a, 2015b).

Nepal is growing wheat in 0.7 million hectares of land on average annually between 2006-2017. The average

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Table 1. Production and Yield of Wheat in Nepal and World (2006-2017)

Year	Area (ha)	Production (Metric ton)	Yield (MT/ha)				
	Nepal	Nepal	Nepal	Bangladesh	New Zealand	Ireland	World
2006	672040	1394126	2.07	1.53	6.90	9.15	2.89
2007	702664	1515139	2.16	1.85	8.50	8.46	2.82
2008	706481	1572065	2.23	2.18	8.11	8.97	3.06
2009	694950	1343862	1.93	2.15	7.49	8.17	3.04
2010	731131	1556539	2.13	2.40	8.12	8.60	2.97
2011	767499	1745811	2.27	2.60	7.29	9.86	3.16
2012	765317	1846142	2.41	2.78	8.92	7.22	3.09
2013	754243	1727346	2.29	3.01	9.11	9.00	3.25
2014	754474	1883147	2.50	3.03	8.63	10.01	3.30
2015	762373	1975625	2.59	3.09	8.67	10.67	3.36
2016	745823	1736849	2.33	3.03	9.20	9.54	3.40
2017	735850	1879191	2.55	3.16	9.86	10.17	3.53
Average	732737	1681320	2.29	2.57	8.40	9.15	3.16

Source: FAO Statistics, 2006 to 2017 (http://www.fao.org/faostat/en/#data/QC)

Table 2. Wheat genotypes used in the study

Entry No.	Genotypes	Source	Parentage	Released Year
1	BL4335	Bhairahawa	n.d	
2	NL1202	Mexico	n.d	
3	NL1207	Mexico	n.d	
4	NL1211	Mexico	n.d	
5	NL1244	Mexico	n.d	
6	NL1247	Mexico	n.d	
7	NL1253	Mexico	n.d	
8	NL1254	Mexico	n.d	
9	BL4699	Bhairahawa	n.d	
10	BL4707	Bhairahawa	n.d	
11	BL4708	Bhairahawa	n.d	
12	NL4307	Mexico	n.d	
13	NL1260	Mexico	n.d	
14	NL1325	Mexico	n.d	
15	NL1326	Mexico	n.d	
16	NL1327	Mexico	n.d	
17	NL1328	Mexico	n.d	
18	BHRIKUTI	Mexico	CMT/COC75/3/PLO//FURY/ANA75	1994
19	RR21	India	1154-388/AN/3/YT54/NIOB/RL64	1971
20	GAUTAM	Nepal	SIDDHARTH/NING8319/NL297	2004

n.d: data not available
(Source: National Wheat Research Programme(NWRP),Bhairahawa, Nepal)

yield is 2.29 tons/ha which is significantly lower than the yield of wheat in Bangladesh (2.57 tons/ha) and the average yield in the world (3.16 tons/ha), and very low compared to the countries with highest yield (New Zealand 8.4 tons/ha; Ireland 9.15 tons/ha) (FAO statistics, 2006 to 2017) (**Table 1**). The limiting factors for the yield of wheat in Nepal appear to be water and genetic makeup of the varieties. The yield of wheat in the rainfed area was 1.74 MT/ha, whereas that in the irrigated area was 2.71 MT/ha. Similarly, the yield from the local seeds was 1.12 MT/ha, whereas that from the improved seeds was 2.34 MT/ha (MoALMC 2018). Wheat is grown as winter crop sown in October or November and harvested in March or April. It is also grown as a summer crop sown in April or May coming to fruition in September or October (Joshi *et al.* 2006). Precipitation and temperature affected the yield of wheat in various ways and appear to be important factors (Poudel *et al.* 2014). Our team has undertaken a study to assess the genetic diversity of the wheat improved for drought and heat resistance and assess the stability of wheat genotypes under experimental drought and heat stress. We present our finding on the genetic diversity of drought and heat resistant varieties assessed by SSR markers.

MATERIALS AND METHODS

Genetic Material

The genetic material used in this study are the seeds of 20 wheat genotypes collected from the National Wheat Research Programme (NWRP), Bhairahawa, Nepal (**Table 2**). From each genotype, seedlings were grown in pots at the glasshouse in 2017. Leaves from 12-14 days old seedling were sampled for DNA extraction taking precaution for contamination.

Genomic DNA Extraction

Genomic DNA from the sampled wheat leaves was extracted following the method described (Yu *et al.* 2017) with minor modification. The final product was quantified spectrophotometrically and a stock solution of various concentration was prepared in nuclease-free water. The integrity and quantity of genomic DNA extract were also checked by electrophoresis of 1 µg DNA on an agarose gel.

Table 3. Scoring for the PCR product band and information regarding the genome related to the SSR marker

SN	Marker	Primer Sequence(F/R)	chromosome	PCR Annealing temperature
		Drought tolerance SSR markers		
1	Wmc54	F: TATTGTGCAATCGCAGCATCTC R: TGCGACATTGGCAACCACTTCT	3B	49
2	Wmc63	F: GTGCTCTGGAAACCTTCTACGA R: CAGTAGTTTAGCCTTGGTGTGA	2A	49
3	Wms06	F: CGTATCACCTCCTAGCTAAACTAG R: AGCCTTATCATGACCCTACCTT	4B	49.5
4	Wms108	F: CGACAATGGGGTCTTAGCAT R: TGCACACTTAAATTACATCCGC	3B	46.5
5	Wms118	F: GATGTTGCCACTTGAGCATG R: GATTAGTCAAATGGAACACCCC	5B	47.5
6	Wms30	ATCTTAGCATAGAAGGGAGTGGG TTCTGCACCCTGGGTGAT	2A	47.5
7	Wms149	F: CATTGTTTTCTGCCTCTAGCC R: CTAGCATCGAACCTGAACAAG	4B	47
8	Wms169	F: ACCACTGCAGAGAACACATACG R: GTGCTCTGCTCTAAGTGTGGG	6A	50.5
9	Wms198	F: TTG AAC CGG AAG GAG TAC AG R: TCA GTT TAT TTT GGG CAT GTG	4A	45.5
10	Wms375	F: ATTGGCGACTCTAGCATATACG R: GGGATGTCTGTTCCATCTTAGC	4B	49
11	Wms304	F: AGG AAA CAG AAA TAT CGC GG R: AGG ACT GTG GGG AAT GAA TG	2A	51
12	Wms135	F: TGT CAA CAT CGT TTT GAA AAGG R: ACA CTG TCA ACC TGG CAA TG	1A	50.5
		Heat tolerance SSR markers		
13	Xbarc197	F: CGCATGGTCAGTTTTCTTTTAATCC R: GCGCTCTCCTTCATTTATGGTTTGTTG	3A	51.5
14	Xbarc84	F: CGCATAACCGTTGGGAAGACATCTG R: GGTGCAACTAGAACGTACTTCCAGTC	3B	54.5
15	Xgwm285	F: ATGACCCTTCTGCCAAACAC R: ATCGACCGGGATCTAGCC	3B	47.5
16	Xbarc217	F: GCGTTGTGTTGAAGGCTGAGCATCCA R:GCGGAGTAGCCTAACGGCGGTGGAGGAAAC	4D	59.5

SSR Marker Primers

Twelve pairs of SSR markers including eight drought tolerance related SSR markers as described (Bousba *et al.* 2012) and four heat stress tolerance SSR markers as described (Alsamadany 2016) were purchased from GCC Biotech (India) Pvt. Ltd (**Table 3**). SSR Primer sequence, wheat genome linked to the SSR marker and the annealing temperature used for the PCR are also indicated. The dry powder of primer received from the vendor was dissolved in nuclease-free water and an aliquot of it was used for making a stock solution for PCR.

PCR

The SSR-PCR reaction mixture was prepared from each genomic DNA and primer pair was prepared in 25ul which contained 50 ng of genomic DNA, 0.5 unit of DreamTaq Polymerase (ThermoFisher Scientific), 0.2mM dNTPs, 1.5 mMof MgCl2 and 10pmolof each primer. The PCR amplification of genomic DNA was started by heating the lid of the thermocycler at 100°C and then incubating the DNA samples at 95°C for 5 min, then 35 cycles comprising 94°C for 20 sec, annealing of primer at 45-60°C (generally at 5° below the average Tm of the primer pair) for 20 sec and then extension at 72°C for 30 sec. The final extension was carried out at 72°C for 2 min in ABI 9700GeneAmp thermocycler (SeqGen).

Electrophoresis and scoring

Electrophoresis of the PCR product was carried out on 1.0%of agarose gel in 1X TBE Buffer containing 0.5 µg/mL Ethidium Bromideat 100 V for 60 Minutes and observed under a UV transilluminator. Bands from each sample were counted and the presence and absence of bands were scored as 1 and0 respectively. The cluster analysis of 20 wheat genotypes was performed using NTSYSpc version 2.02. Scoring is made visually and with the help of the software GelAnalyzer 2010 for the total number of bands (TB), polymorphic bands (PB) and determination of the size of amplicons. Allelic variation and the polymorphism information content (PIC) was analyzed using PowerMarker 3.25 (https://brcwebportal.cos.ncsu.edu/powermarker).

RESULT

PCR and Scoring

Examples of gel electrophoresis of SSR-PCR products are shown in **Figs. 1A** and **1B**. **Fig. 1A** shows the PCR products from 20 genotypes of wheat obtained with the SSR primer pair Xbarc197 and **Fig. 1B** shows the PCR products obtained with the SSR primer pair Wms108. Scoring for the PCR product band and information regarding the genome related to the SSR marker are shown in **Tables 3**. 12 SSR markers for drought tolerance gave a total of 29 amplicons. All of them were polymorphic. Two of the markers, Wms304 and Wms135 did not produce any amplicon. The percentage of polymorphism ranged from 75% to 100% with an average of 97.5%. The polymorphic Information Content (PIC) value varied from 0.144 for Wmc63 to

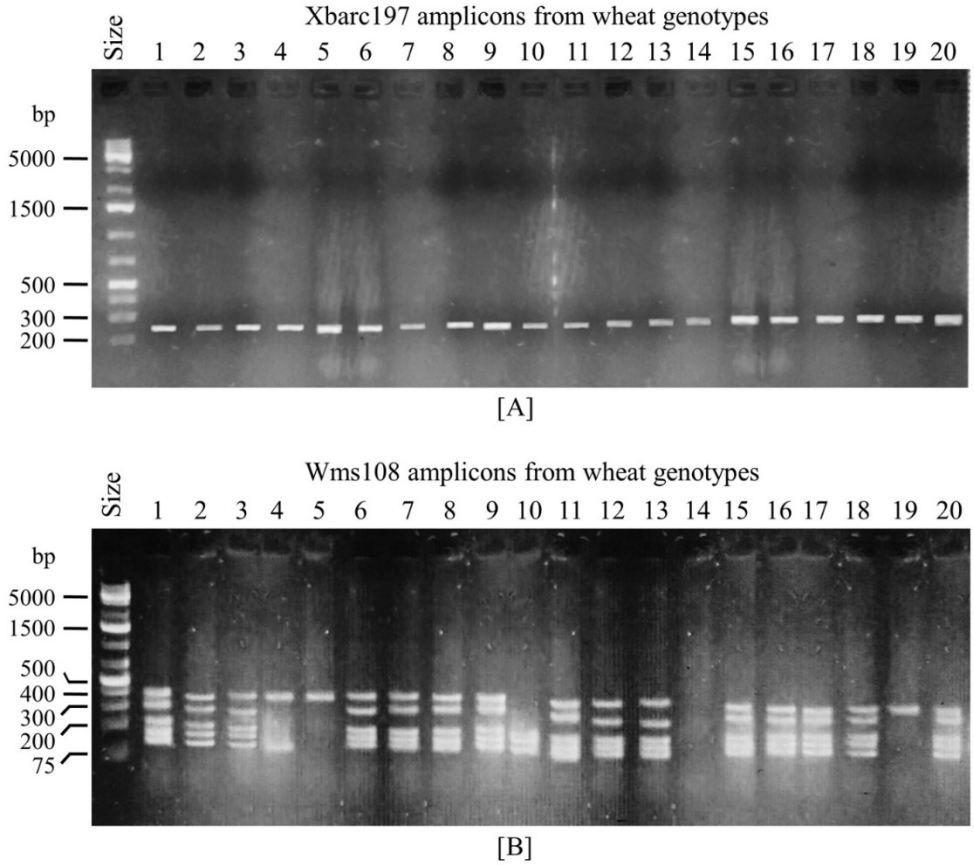

Fig. 1. Amplicons from 20 wheat genotypes with SSR-PCR. [A]Amplicons from 20 wheat genotypes with primers for SSR marker Xbarc197. [B]Amplicons from 20 wheat genotypes with primers for SSR marker Wms108. List of wheat genotypes is shown in **Table 2**

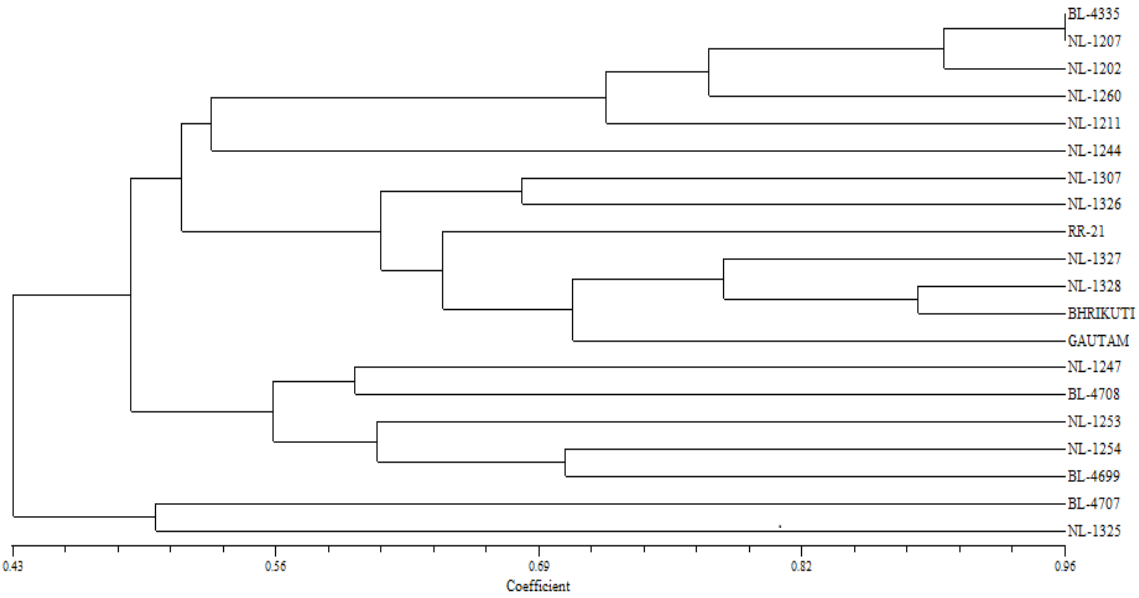

Fig. 2. Dendrogram of wheat genotypes based on amplicons of drought tolerance related SSR markers

0.73 for Wms118 with an average of 0.392. Similarly, 4 SSR markers for heat tolerance gave a total of 5 amplicons. All of them were polymorphic. The percentage of polymorphism ranged from 50% to 100% with an average of 87.5%. The polymorphic Information Content (PIC) value varied from 0.437 for Xbarc84 and Xbarc217 to 0.51 for Xgwm285 with an average of 0.471.

Table 4. List of SSR markers and their PCR amplicons

	Marker	Band size range	Total Bands	Polymorphic Bands	% of polymorphism	Polymorphic Index Content
			[A] Drought tolerance SSR markers			
1	Wmc54	142-510	2	2	100	0.371
2	Wmc63	100	1	1	100	0.144
3	Wms06	66-205	4	3	75	0.42
4	Wms108	100-400	5	5	100	0.378
5	Wms118	75-900	4	4	100	0.738
6	Wms30	100	1	1	100	0.43
7	Wms149	180-600	3	3	100	0.469
8	Wms169	200-220	2	2	100	0.160
9	Wms198	100-700	5	5	100	0.633
10	Wms375	500-600	2	2	100	0.174
11	Wms304		0			
12	Wms135		0			
	TOTAL		29	28		
	AVERAGE		2.9	2.8	97.5	0.392
			[B] Heat tolerance SSR markers			
13	Xbarc197	136-250	2	1	50	0.5
14	Xbarc84	123	1	1	100	0.437
15	Xgwm285	223	1	1	100	0.51
16	Xbarc217	102	1	1	100	0.437
	TOTAL		5	4		
	AVERAGE		1.25	1	87.5	0.471

Table 5. Clusters identified from the dendrogram based on the amplicons of drought tolerance related SSR markers

Cluster	Genotypes	Similarity (%)	Sub-cluster	Genotypes	Similarity (%)
1	BL-4707, NL-1325	50% with each other			
2	NL-1247, BL-4708, NL-1253, NL-1254, BL-4699	49% with cluster 3 and 4	2A	NL-1254, BL-4699	70% with each other
			2B	NL-1253	61% similar with 2A
			2C	NL-1247, BL-4708	60% with each other
3	NL-1307, NL-1326, RR-21, NL-1327, NL-1328, BHRIKUTI, GAUTAM	61% with each other	3A	NL-1327, NL-1328, BHRIKUTI, GAUTAM	70% with each other
			3B	RR-21	64% similar with 3A
			3C	NL-1307, NL-1326	68% with 3B
4	BL-4335, NL-1207, NL-1202, NL-1260, NL-1211	52% with each other	4A	NL-1211	72% similar with 4B
			4B	BL-4335, NL-1207, NL-1202, NL-1260	78% similar with each other

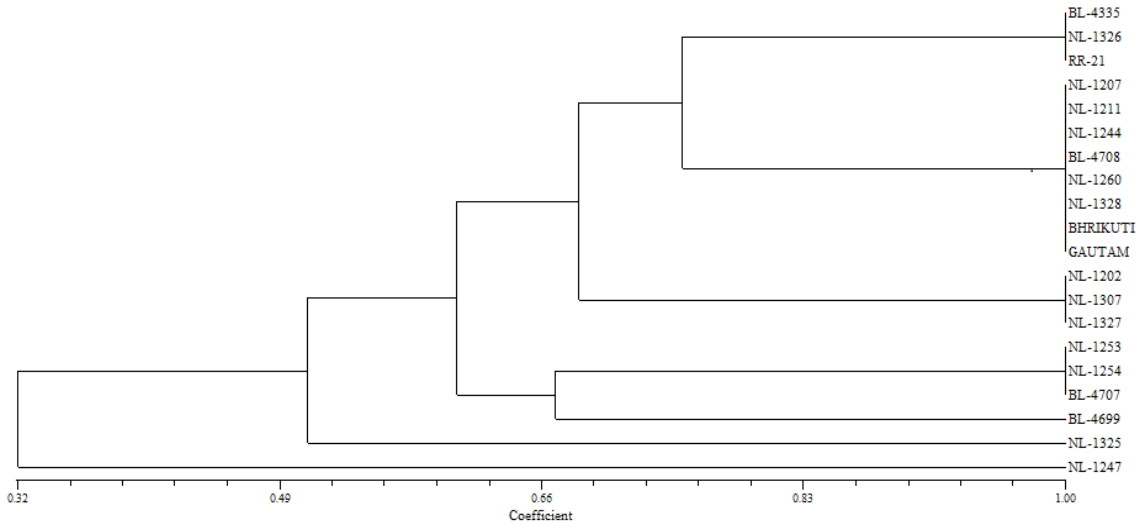

Fig. 3. Clusters identified from the dendrogram based on the amplicons of heat stress tolerance related SSR markers

Cluster Analysis

The result of the cluster analysis of Nepalese wheat genotypes based on the polymorphism of SSR marker for drought tolerance are presented in **Figs. 2** and **3**, and **Table 4**. The result from the SSR markers for drought tolerance is presented in **Fig. 2** and **Table 5**. The SSR markers for drought tolerance have divided 20 genotypes into 4 broad clusters. Three of them could be divided into 8 sub-clusters. The first cluster consisting of BL-4707 and NL-1325 were most divergent from the rest of the genotypes. The SSR markers for heat stress tolerance have divided 20 genotypes into 5 broad clusters. Two of them (cluster 3 and 5) could be divided into 4 sub-clusters. Genotypes within subclusters generated by SSR markers for heat stress tolerance were highly similar.

Table 6. Identifiable clusters from the dendrogram based on amplicons of heat stress tolerance related SSR markers

Cluster	Genotypes	Similarity	Sub-cluster	Genotypes	Similarity
1	NL1247	32 with remaining genotypes			
2	NL1325	50% with cluster 3			
3	NL1253, NL1254, BL4707, BL4699	67% with each other	3A	BL4699	
			3B	NL1253, NL1254, BL4707	100% similar with each other
4	NL1202, NL1307, NL1327	67% with cluster 1			100% similar with each other
5	BL4335, NL1326, RR21, NL1207, NL1211, NL1244, BL4708, NL260, NL1328, BHRIKUTI, GAUTAM		4A	NL1207, NL1211, NL1244, BL4708, NL260, NL1328, BHRIKUTI, GAUTAM	100% similar with each other
			4B	BL4335, NL1326, RR21	100% with each other

DISCUSSION

The productivity of wheat in Nepal is significantly lower than the global average and very low compared to countries with the highest productivity, and it has remained more or less stagnant in the last ten years (**Table 1**). Nepal is highly vulnerable to climate change and crop yield has taken a toll (Marahatta *et al.* 2009, Poudel *et al.* 2014). These call for, among other measures, breeding for drought and heat stress tolerant wheat and other crops. The present study which assesses the genetic diversity among the drought and heat stress tolerant varieties of wheat in Nepal provides useful genetic information for breeding and study of the interaction of genotypes and environment. This study has established the usefulness of 8 SSR markers for drought tolerance and 4 SSR markers for heat stress tolerance to assess the genetic diversity of 20 varieties of wheat released or in the pipeline in Nepal. The result obtained from the present study is in agreement with a similar study done elsewhere (Alsamadany 2016, Bousba *et al.* 2012, Khanjari *et al.* 2007).

The set of drought tolerance linked SSR markers clustered the 20 genotypes into 4 major clusters or 9 sub-clusters with a high degree of polymorphism among individuals within the subcluster. On the other hand, the set of heat stress tolerance linked SSR markers clustered the 20 genotypes into 5 major clusters or 7 sub-clusters with a high degree of similarity among individuals within the subcluster. One genotype, namely NL-1247, was found to be an outlier. This genotype most distantly related to other genotypes assessed in this study is an interesting genotype and has a potential to be useful in the breeding program particularly in wheat hybridization programme either to develop productive recombinant or to exploit heterosis. The information on genetic variation and clustering is also useful to study the genetic stability of wheat varieties under various abiotic stresses and experimental conditions and identify genes and mechanism involved in stress susceptibility and tolerance. Our team has undertaken a study to assess the genetic diversity of the wheat improved for drought and heat resistance and assess the stability of wheat genotypes under experimental drought and heat stress and we expect to report a study in this line.

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