IDENTIFICATION OF QTLs ON CHROMOSOME 1B FOR GRAIN QUALITY TRAITS IN BREAD WHEAT (*TRITICUM AESTIVUM* L.)

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The present study was designed considering the importance of grain quality traits, genetic diversity and marker-trait association analysis in wheat. A significant amount of genetic diversity was found for various seed traits though the genotypes included in the study were found structured. The extent of polymorphism was high with a range of 2–13 alleles and average of 6.5 alleles per locus. Population structure was detected with 30 unlinked SSRs that divided the population of 92 genotypes in three sub-populations. Extensive LD extent was found on chromosome 1B with 42 SSRs specific for 1B chromosome. Marker-trait associations were determined using mixed linear model, where, population structure and kinship calculated on the basis of unlinked markers were covariated with 1B specific markers and traits data. Eight QTLs for five traits including protein, gluten contents, test weight bread and chapati making quality. Protein content, test weight, bread quality and Glu-B1 were found significantly associated with primers WMC419 (32 cM); WMC128 (30 cM), WMC419 (32 cM); WMC818 (17 cM) and WMC416 (44 cM), respectively.

Keywords: *association mapping, linkage disequilibrium, population structure, quality traits, wheat.*

Introduction. Genetic diversity prevailing in the germplasm can be exploited in the best way by tagging traits of economic importance with molecular markers [1]. In wheat, 40 traits of economic importance have been associated with molecular markers [2]. Knowing the position of nucleotide sequence underlying a specific trait offers plant breeders an opportunity to apply marker assisted selection. Most of quality traits are controlled by many loci and their molecular characterization and genetic mapping is called quantitative trait loci mapping (QTL-mapping). Family based linkage analysis and LD based association mapping are two of the most commonly used tools for QTL mapping [3, 4]. In linkage analysis, genes are assigned on different chromosomes on the basis of co-inheritance of markers in a segregating population. The limitations of linkage analysis are development of bi-parental population, limited allele coverage, low resolution, marker specificity, longer time period and high cost [1, 5]. A relatively new approach called association mapping overcomes these limitations by using natural germplasm and historical recombination and mutations. Association mapping yields high-resolution, broader allele coverage, and cost effective gene tagging [6]. After its first application for milling quality in 2006, association mapping studies in hexaploid wheat are being extended to tag yield traits, protein quality, and tolerance to biotic and abiotic stresses [2]. Most of these studies have focused on individual chromosomes, where, QTLs have been previously identified by FBL-mapping approach [7–9]. Covering the whole genome of hexaploid wheat with sufficient mapping resolution requires hundreds of SSR markers, therefore, targeting individual linkage group is a reasonable strategy as adopted by Yao et al. [10] and Liu et al. [11].

The results of association mapping in the current study confirmed some previous reports in addition to detecting novel QTLs. The markers which were found significantly linked to the seed traits may be utilized for the improvement of these traits through marker assisted selection (MAS).

Materials and Methods. *Phenotyping.* For genotyping 92 accessions were selected on the basis of diversity in phenotypic data recorded for various seed and flour traits (Table S1, http://cytgen.com/articles/5020013s.pdf). Fourteen traits including protein, moisture, starch, gluten, 1000-grain wt., test wt., bread quality, chapati quality (Chq), seed length, seed width, seed thickness, grain hardness, flour pH, yield per plant (YPP). Ten guarded and tagged plants from each plot were harvested and grains were weighed in grams. Total grain weight

was divided by number of plants harvested to estimate grain yield per plant. The length, width and thickness of 20 grains taken randomly from each wheat accession were measured using micrometer. Thousand kernel weight was recorded in grams per 1000 kernels. 1000 grains were counted from the harvest of each plot and weighed to determine 1000 kernel weight of each genotype.

Near Infrared Reflectance (NIR) spectroscopy. The test weight (kg/hL) of each genotype was recorded according to the method given in AACC (2000) method no. 55-10. A 1 liter vessel was overfilled with wheat grains. Then it was levelled and weighed to get the test weight of the respective wheat accession. Each wheat accession was tested for grain hardness by running it through NIR analyzer Inframatic 8620 (Perten Instruments, Inc. IL, USA) according to the procedure described in AACC (2000) method no. 39-70 A. The data on protein content, moisture content, starch content and gluten content were recorded using near infrared reflectance (NIR) spectroscopy by running the samples through (Inframatic 9100, Perten instruments, Inc. IL) according to the procedure described in AACC (2000) method no. 39-11. The pH of each flour was determined by using pH meter according to the procedure mentioned in AACC (2000) method no. 2-52. The dough for chapatti was made by mixing 200 g of whole meal wheat flour with predetermined quantity of water for 3 min and allowed to rest for 30 min. A dough piece weighing 80 g was rolled on a sheet of 2 mm thickness with a wooden roller pin on a specially designed wooden platform and cut into circle of 17 cm diameter. The chapattis were baked on thermostatically controlled hot plate at a temperature of 210 °C for 1.5 min. Sensory evaluation of chapattis was carried out for color, flavor, taste and texture using hedonic scale by a panel of trained judges. The breads were prepared according to the AACC (2000) straight dough method no 10-10B. The ingredients were mixed for 5–10 min in a Hobart A200 Mixer to form dough and allowed to ferment at 30 °C and 75 % R.H. for 180 min. First and second punches were made after 120 and 150 min, respectively. The dough was molded and panned into 100 g test pans, and final proofing was done for 45 min at 95 °F (35 °C) and 85 % R.H. The bread was baked at 232 °C for 13 min. The sensory scores for external characteristics (volume, crust color, symmetry, evenness of bake, break and shred) and for internal characteristics (grain, crumb color, aroma, taste, mastication and texture) were recorded for each loaf assigned by a panel of trained judges according to the bread score method developed by the American Institute of Baking and reported by Matz [12].

Genotyping and PCR reaction. DNA from a fresh leaf tissue of each genotype was extracted following the method described by Weining and Langridge [13] with few modifications. A total of 30 unlinked SSR markers covering all chromosomes were selected from GrainGenes data base (http://wheat.pw.usda.gov) and used for detecting population structure. Based on the consensus map Ta-SSR-2004 [14] 42 SSR markers on chromosome 1B were used to determine linkage disequilibrium (LD) and marker-trait association (MTAs). All PCR runs were performed with a negative control missing template DNA and a positive control with Chinese Spring DNA. The volume of each PCR reaction was 20 μl, therefore 18 μl of master mix was pipette to each well of PCR plate containing 2 μl of template DNA. The composition of master mixture was as follow:

10× PCR buffer	196 μl (2 μl/reaction)
25 mM $MgCl_2$	156.8 μl (1.6 μl/reaction)
0.2 mM dNTPs mix.	627.2 μl (6.4 μl/reaction)
5 μM Forward primer	98 μl (1 μl/reaction)
5 μM Reverse primer	98 μl (1 μl/reaction)
DEPC water	568.4 μl (5.8 μl/reaction)
Taq DNA polymerase	19.6 μl (0.2 μl/reaction)
Total volume	1764 μl (18 μl/reaction)

The PCR profile for each SSR primer pair and sequence are given in (Table S2, http://cytgen.com/articles/5020013s.pdf). PCR products were analyzed in 2.5 % high resolution agarose according to manufacturer's instructions.

Statistical analysis. Summary statistics for all seed traits were calculated using the software Statistica v7. The analysis of variance for augmented design was performed on the data collected for fourteen seed traits for two consecutive years i.e., 2009–2010 and 2010–2011. Population structure was detected with unlinked SSRs. LD extent was found on chromosome 1B with 41 SSRs specific for 1B chromosome (Table S3, http://cytgen.com/articles/5020013s.pdf). Markers polymorphic on 2.5% high resolution agarose were used for statistical analysis of population structure, LD and marker

trait associations. Polymorphism and population structure was calculated following the procedure commonly used by researchers [7]. Linkage disequilibrium between all pairs of loci was estimated using LD parameter r^2 (the squared correlation coefficient between all bi-allelic combinations at two loci and summarizes both recombination and mutational history. Both unlinked and syntenic r^2 were evaluated using the software TASSEL 3 (http://www.maizegenetics.net) by setting 1000 permutation. The 95 % percentile of unlinked r^2 was used to derive a critical value for r^2, as evidence of linkage [7]. The loci having $P < 0.001$ were considered to be in significant LD. If all pairs of flanking loci within a chromosomal region were in significant LD, the region was designated as LD block [7]. The marker-trait association analysis was performed using the software TASSEL v3 and mixed linear model (MLM) as this model can reduce both type I and type II errors. The MLM simultaneously co-variated kinship and population structure with marker-trait association tests. Kinship matrix was calculated using unlinked markers by TASSEL 3 and population structure by program STRUCTURE 2.2. The significance of marker-trait associations was declared by $P \leq 0.01$ and the magnitude of QTL effects was calculated by R^2-marker.

Results. *Analysis of variance (ANOVA).* The genotypes included in the study showed significant variation for most of the traits. All the traits except moisture content and flour pH showed a statistically significant variation in the germplasm. Genotype × year interaction was significant only for 1000-grain weight and yield per plant. The block effect was also significant for most of the quality traits while it was non-significant for moisture, seed length, seed width, seed thickness, grain hardness, flour pH and grain yield (Table S4, http://cytgen.com/articles/5020013s.pdf).

The range for protein percentage was 11.1 to 15.3 % during 2009–2010 and 11.0–16.0 % during the year 2010–2011 (Table 1 & 2). Moisture had a range from 10.4 to 13.2 % for the year 2009–2010 and 10.5 to 12.9 % for the year 2010–2011. The range for starch was 49.3 to 56.6 % for the year 2009–2010 and 50.9 to 58.2 % for the year 2010–2011. The minimum and maximum values of gluten for the years 2008–2009 and 2009–2010 were 18 to 30 % and 15 to 33 % respectively (Table 1). The range of thousand grain weight for the years 2009–2010 and 2010–2011 was 22.0–44.5 g and 22.5–46.1 g respectively. The test weight ranged from 68.50 to 78.90 kg/hl during 2009–2010 and 66.75 to 79.89 kg/hl during 2010–2011.The range of bread quality, chapatti quality, seed length, seed width, seed thickness, grain hardness, flour pH and grain yield per plant was 62.0–80.0, 1.0–6.0, 5.47–6.91 cm, 2.73–3.50 cm, 2.15–3.12 cm, 62.25–72.33, 6.00–6.39 and 13.30–63.37 g during 2009–2010 and 62.0–80.37, 1.0–6.0, 5.09–7.32 cm, 2.59–3.79 cm, 1.99–3.34 cm, 60.74–74.22, 5.97–6.41 and 12.32–60.98 g respectively during 2010–2011 (Table 2).

Marker allele diversity. Out of 81 SSRs used 72 (88.89 %) were polymorphic on 2.5% high resolution agarose. Only polymorphic SSRs were used for statistical analysis. The extent of polymorphism was high with a range of 2–13 alleles and average of 6.5 alleles per locus. The resulted allelic diversity indicated significant diversity in the studied genotypes.

Population Structure. The admixture model-based analysis with software STRUCTURE identified an optimal number of sub-populations when K was set at 3, because likelihood peaked at $K = 3$ in the range of one to twenty sub-populations

Table 1. **Descriptive Statistics for year-1**

Trait names	Min.	Max.	Mean	Std. Deviation
Protein (%)	11.10	15.30	13.21	0.85
Moisture (%)	10.60	109.0	12.11	8.19
Starch (%)	51.70	56.60	53.98	0.97
Gluten (%)	19.00	30.00	24.32	2.78
1000 grain wt. (g)	23.00	44.40	34.20	4.33
Test wt. (Kg/hL)	70.50	78.30	74.76	1.52
Bread quality	62.00	80.00	71.33	3.45
Chapati quality	1.00	6.00	3.76	1.03
Seed length (mm)	5.54	6.82	6.32	0.27
Seed width (mm)	2.73	3.50	3.09	0.14
Seed thickness (mm)	2.15	3.10	2.73	0.18
Grain hardness	62.25	72.23	66.79	2.11
Flour pH	6.00	6.39	6.21	0.12
YPP (g)	13.30	63.37	36.64	8.85

Fig. 1. Population structure of 92 wheat genotypes in order of Q. The population was assigned to three color-coded sub-populations. Each bar represents single genotype and the colored portions in each bar reveal the proportional contribution of each sub-population to that genotype

(Fig. 1). The likelihood showed clear peaks $k = 13$ and $k = 18$ as well but according to Evanno et al. [15], the minimum value of k that showed a peak was picked as the actual value of k. The number of genotypes assigned to each inferred sub-population ranged from 25 to 40 (Fig. 1). The F_{ST} values between all sub-populations were significant ($P < 0.001$) confirming the real difference among these sub-populations and presence of genetic structure.

Linkage Disequilibrium. The unlinked markers used to detect population structure were also used to determine background LD (unlinked LD). The background LD in the genome caused by genetic structure was used to set a critical value of LD for markers on chromosome 1B. The unlinked r^2 value ranged from 0.000 to 0.245 for all unlinked loci pairs with an average of 0.0032. The 95th percentile of the distribution of unlinked r^2 was used as population specific threshold for this parameter as an evidence of LD because of linkage [7] and that was 0.021. The syntenic r^2 was obtained from the analysis of 41 SSRs on chromosome 1B. The value of pairwise syntenic r^2 ranged from 0.00057 to 0.278 with an average of 0.019, significantly higher than the average of unlinked r^2.

Table 2. **Descriptive Statistics for year-2**

Trait names	Min.	Max.	Mean	Std. Deviation
Protein (%)	10.60	15.80	13.25	0.93
Moisture (%)	10.15	12.45	11.33	0.46
Starch (%)	50.65	57.55	54.19	1.39
Gluten (%)	16.00	32.00	24.51	3.08
1000 grain wt. (g)	24.30	44.30	33.86	4.21
Test wt. (Kg/hL)	69.12	79.19	74.88	1.98
Bread quality	62.00	80.68	71.37	3.48
Chapati quality	1.00	6.00	3.79	1.00
Seed length (mm)	5.09	7.32	6.41	0.47
Seed width (mm)	2.63	3.80	3.14	0.24
Seed thickness (mm)	1.99	3.40	2.75	0.28
Grain hardness	61.33	73.60	66.80	2.40
Flour pH	5.972	6.40	6.20	0.11
YPP (g)	12.93	60.98	36.45	8.92

Marker-Trait associations. Mixed linear model approach was used to determine marker-trait associations for fourteen seed traits and 42 SSRs on 1B. Similar results were obtained for both years. A total of six SSRs were identified to be associated with five traits at the probability level of 0.01 (Table 3). Gluten content was significantly associated with markers XPSP3000 (0 cM) and GWM374 (18 cM). The primers XPSP3000 and WMC416 were significantly associated with chapatti quality. Protein, test weight, bread quality and Glu-B1 were found significantly associated with primers WMC419 (32 cM); WMC128 (30 cM), WMC419 (32 cM); WMC818 (17 cM) and WMC416 (44 cM), respectively.

Discussion. *Analysis of variance*. Analysis of variance (ANOVA) showed that a considerable diversity was present within the population for almost all the traits. The blocks were also significantly different from each other for most of the grain quality traits especially protein, starch and gluten contents and ultimately for chapatti and bread quality. This reflects the influence of environment on the grain quality traits. Environmental conditions are known to have a significant influence

on end-use quality characteristics of wheat, but the relative magnitude of environmental, genetic and G × E effects on quality is unclear [16]. Protein content, which is the most important trait in quality evaluation and breeding of durum wheat, is known to be influenced mainly by environment, cultivar, nitrogen fertilizer rate and time of nitrogen application [17].

The range for protein is comparable with protein (12 to 17.7 %) found in three wheat populations grown in Aberdeen [18]. Miralbes [19] reported NIR moisture content ranging from 9.32 to 17.58 %. The extent of protein and gluten contents is supported by the findings of Dencic et al. [20] in 140 genotypes of 28 different countries. The extent of test weight is in line with that reported by Martin et al. [21], who reported a range of 66.2–80.2 kg m^{-3} in 130 hard red spring wheat recombinant inbred lines.

Marker allele diversity. The extent of polymorphism was high with a range of 2–13 alleles and average of 6.5 alleles per locus. In the present study, the number of alleles per locus for SSRs was found to 6.5 which is comparable to 5.62 reported 94 Pakistani genotypes by Sajjad et al. [22]. It is higher than 5.7 reported in 108 Chinese wheat genotypes by Yao et al. [10] and 4.8 reported in a collection of 95 Eastern USA soft-winter cultivars [7]. It is less compared to 18.1 in 998 accessions of hexaploid wheat from IPK gene bank [23].

Linkage Disequilibrium. The prevalence of LD in the targeted genomic region is pre-requisite for association mapping. A review of previous reports on LD extent indicates that LD extent varies with varying wheat populations [2]. Chao et al. [24] examined genome wide LD among 43 US cultivars using 242 SSRs distributed throughout the genome. For this germplasm genome wide LD decayed to 0.2 within a distance of 10 and 20 cM. This LD extent is much higher compared to that reported by Berseghello and Sorrels [7]. Tommasini et al. [9] surveyed 91 SSRs and STS markers in 44 varieties and 240 RILs. They observed LD on chromosome 3B, extended up to 0.5 cM in 44 varieties and in 240 RILs; it was extended up to 30 cM. This study favors the usefulness of germplasm over bi-parental populations in association mapping. Sajjad et al. [22] observed LD extent up to ~ 40 cM ($r^2 = 0.015$) on chromosome 3A in 94 spring wheat genotypes including mostly Pakistani

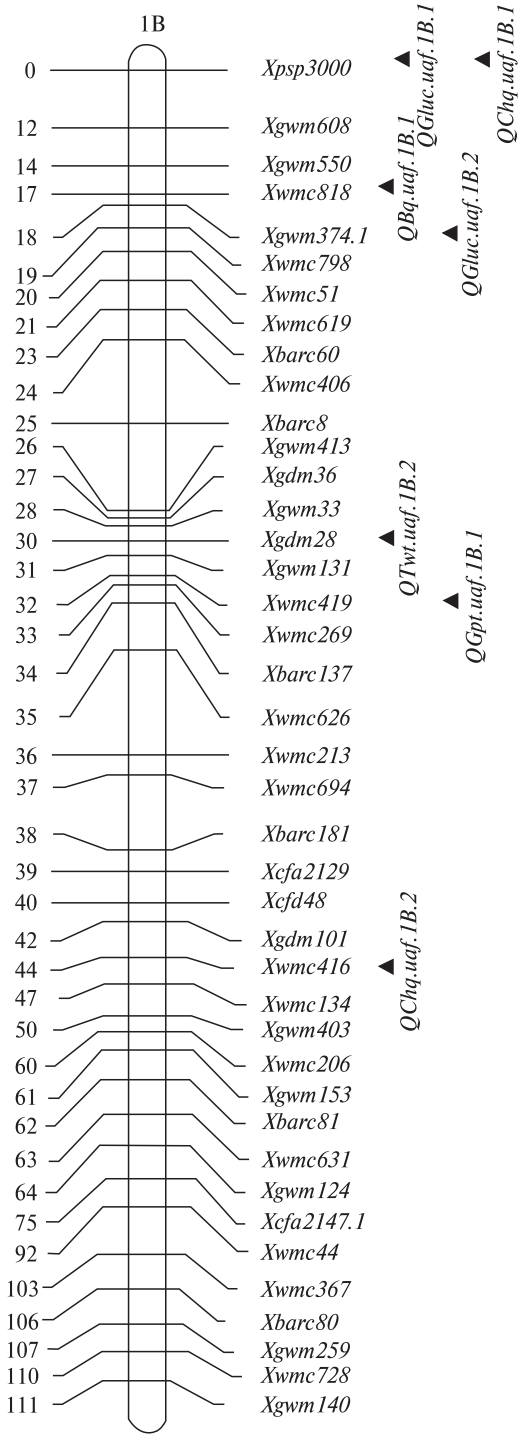

Fig. 2. Genetic linkage maps of wheat chromosome 1B. The locations of QTLs for bread quality (Bq), Gluten (Gluc), Chapati quality (Chq) and Test weight (Twt) identified by MLM association mapping approach are shown on map

Table 3. **Association (R^2) of the SSR markers with seed traits in wheat**

Trait	QTL	Marker	Map position (cM)	R^2 (%)	
				2009–2010	2010–2011
Protein	*QGpt.uaf.1B.1*	WMC419	32	5.88	6.30
Gluten	*QGluc.uaf.1B.1*	XPSP3000	0	4.74	4.06
	QGluc.uaf.1B.2	GWM374	18	2.95	3.25
Test weight	*QTwt.uaf.1B.1*	WMC128	30	4.19	4.15
	QTwt.uaf.1B.2	WMC419	32	2.88	4.21
Bread quality	*QBq.uaf.1B.1*	WMC818	17	3.53	4.35
Chapatti quality	*QChq.uaf.1B.1*	XPSP3000	0	3.78	3.80
	QChq.uaf.1B.2	WMC416	44	3.60	2.84

Note. Map position (cM) were based on the consensus map Ta-SSR-2004 [14]; R^2 indicates the percentage of the total variation explained at the loci significant at $P < 0.01$ level.

landraces, cultivars and elite lines surveyed with 23 polymorphic SSRs. Zegeye et al. [25] also observed lower LD ($r^2 = 0.116$) with SNPs on 1B as compared to LD with SSRs in this study. These reports on LD structure in hexaploid wheat reveal that LD structure varies with populations, genomic regions and type of marker.

Marker-trait associations. Mixed linear model approach was used to determine marker-trait associations for fourteen seed traits and 41 SSRs on 1B. The mixed linear model (MLM) is more powerful as compared to general linear model (GLM) or any other model developed so far. The mixed linear model (MLM) approach was used by many researchers to find marker trait associations in wheat [2, 22]. Ravel et al. [8] genotyped 113 diverse genotypes with SNPs to identify the best gene underlying Glu-1Bx of the two previously reported candidate genes (Glu-B1-1 and spa-B) through family-based linkage analysis [26]. No significant LD was observed between these two candidate genes and significant MTA was found between one haplotype of Glu-B1-1 and an increase in quantity of Glu Bx. This study also demonstrated the potential utility of association mapping and its discriminating power between two closely linked candidate genes. Neumann et al. [27] detected major QTLs for protein content and sedimentation volume in a world-wide panel of 96 winter wheat accessions using association mapping. The position of one of the QTLs for protein on 4B was analogous to that of *Qpro.mbg*-4B and the position of *Qsev.mbg*-7A detected as MTA for sedimentation volume was similar to that of the QTL previously detected by Blanco et al. [28], [29], respectively, through family based linkage analysis. Marker-trait associations (MTAs) for 1,000 grain weight, protein content, and sedimentation volume with 115 SSRs were tested in a collection of 207 diverse soft winter wheats using association mapping. The SSR *wmc419*-1B was identified at 31.9 cM as a main effect QTL for protein content in studies reported by Reif et al. [30] and Mann et al. [31]. The SSRs namely *barc149*-1D and *taglgap*-1B at 0.0 cM were found linked with genes corresponding to test weight [30]. For these traits, genotypic variance for the underlying QTLs was above 40 % as determined by previous linkage analysis studies [32, 33].

Conclusion. In conclusion, the study successfully identified novel marker trait associations. Marker traits associations identified for bread and chapati making quality would help to improve our understanding about dough processing quality. The detection of major grain quality traits on chromosome 1B shows its importance for breeding programs focusing on grain quality improvement. Further, it can be implicated that focusing on 1B homeologs such as 1A and 1D will be useful in broadening our knowledge about the genetics of grain quality.

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Настоящее исследование проведено с учетом важности признаков качества зерна, генетической изменчивости и анализа соответствия маркер – признак у пшеницы. Показано значительное генетическое разнообразие по различным признакам семян, хотя генотипы, задействованные в работе, были структурированными. Степень полиморфизма была высокой с размахом 2–13 аллелей и в среднем 6,5 аллелей на локус. Популяционная структура оценена по 30 несвязанным SSRs, которые разделили популяцию из 92 генотипов на три субпопуляции. Высокая степень LD обнаружена у хромосомы 1B по 42 SSRs, специфичным для хромосомы 1B. Соответствие маркер – признак определено с использованием смешанной линейной модели, где популяционная структура и подобие, вычисленное на основе несвязанных маркеров, ко-варьировали с 1B-специфичными маркерами и данными признаков. Показано, что содержание белка, тестовой массы, качества хлеба и Glu-B1 значительно ассоциировано с праймерами WMC419 (32 cM); WMC128 (30 cM), WMC419 (32 cM); WMC818 (17 cM) и WMC416 (44 cM) соответственно.

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