https://doi.org/10.17221/121/2017-CJGPB

Construction of a Genetic Linkage Map of Cigar Tobacco (*Nicotiana tabacum* L.) Based on SSR Markers and Comparative Studies

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Abstract

Tong Z., Xiao B., Chen X., Fang D., Zhang Y., Huang C., Li Y. (2018): Construction of a genetic linkage map of cigar tobacco (*Nicotiana tabacum* L.) based on SSR markers and comparative studies. Czech J. Genet. Plant Breed., 54: 115–122.

Genetic linkage maps representing the tobacco genome have been an important tool for breeding programs because of the elucidation of polygenic traits. We constructed a genetic linkage map of cigar tobacco (*Nicotiana tabacum* L.) based on an inter-type backcross population of 213 individuals and performed a comparative analysis with other published maps of dark tobacco and flue-cured tobacco. The map consisted of 562 SSR loci distributed on 24 tentative linkage groups and spanned a total length of 1341.18 cM with an average distance of 2.39 cM between adjacent markers. The comparative analysis revealed a Spearman correlation index of 0.93 for marker order conservation with the previously published maps constructed for different tobacco types. Approximately 91% of the SSR markers common to other inter-type maps were located in the same positions as in previous maps. The three maps exhibit good synteny in terms of the shared markers, which suggests that there might be no translocation variations between the genomes of the cigar, dark and flue-cured tobaccos. These results indicate the feasibility of generating a unique genetic map of preferred traits in cigar tobacco and that such mapping may be helpful for breeding programs because plants derived from different inter-type populations can be rapidly scanned using the markers associated with useful cigar traits.

Keywords: common tobacco; comparative mapping; genetic map; microsatellite marker

The genus *Nicotiana* is a member of the family Solanaceae and has been divided into three subgenera containing over 64 recognized species (Goodspeed 1954). The best-known species is *Nicotiana tabacum* L., which is grown commercially in at least 100 countries around the world (Gerstel *et al*. 1979, 1980). At present, seven types of tobacco are grown commercially including flue-cured, burley, cigar, oriental, sun/air-cured, dark, and other tobaccos, which are defined to a large extent by the region and/or area of production, method of curing, and intended use in manufacturing, as well as by distinct morphological characteristics and chemical differences (Wernsman 1987). Furthermore, cigar tobacco is one of the most important commercial types used for tobacco production in the world, and genetic research on cigar tobacco cultivars such as linkage map construction and comparative studies, is important not only for the mapping of important traits but also for genomics-based breeding purposes.

The development of linkage maps covering the whole tobacco genome has provided new opportunities for the elucidation of complex polygenic traits, enabling tobacco geneticists to identify and manipulate the genetic information necessary to obtain superior genotypes in breeding programs. With the rapid development of next-generation sequencing technologies, tobacco genome sequences have become available to the public through two independent research groups (Bombarely *et al*. 2012; Sierro *et*

https://doi.org/10.17221/121/2017-CJGPB

al. 2013, 2014; Edwards *et al*. 2017). Recently, based on this genome information, many inter- and intra-type tobacco genetic maps have been reported using molecular markers such as DArT (Lu *et at*. 2013), SSR (Bindler *et al*. 2007, 2011; Tong *et al*. 2012, 2016), and SNP (Gong *et al*. 2016). In particular, because of their ubiquitous presence in the genome, highly polymorphic nature, and co-dominant inheritance, SSR markers are among the most popular markers of choice for constructing genetic linkage maps in many tobacco types including dark tobacco (Bindler *et al*. 2007, 2011), flue-cured tobacco (Tong *et al*. 2012, 2016), and burley tobacco (Cai *et al*. 2009). However, in cigar tobacco, only one report of the development of a genetic map was created to identify QTLs for black shank (*Phytophthora nicotianae*) resistance in an inter-type cross (Vontimitta & Lewis 2012).

The objective of this work was to construct a saturated genetic linkage map of cigar tobacco based exclusively on co-dominant SSR markers, and to compare the current map with that of dark tobacco and the previously published map of flue-cured tobacco. Moreover, we tested the hypothesis that sufficient significantly conserved linkage groups and marker order arrangement exist between maps constructed from inter-type crosses to allow the construction of a unique BC_1F_1 genetic map using a common cigar donor parent in combination with different flue-cured cultivars as recurrent parents.

MATERIAL AND METHODS

Plant materials. Flue-cured tobacco cultivar Y3, cigar tobacco line Beinhart1000-1 and F_1 (Y3 × Beinhart1000-1) were used to detect polymorphisms of all the SSR markers. Y3 is a landrace variety originating from Zimbabwe with excellent quality and no resistance to black shank (*P. nicotianae*) and brown spot (*Alternaria alternate*), whereas Beinhart1000-1 is a commercial cigar tobacco cultivar with excellent agronomic traits and high resistance to black shank (both race 0 and race 1 of *P. nicotianae*) and brown spot. To improve the performance of Y3 via backcrossing and marker-assisted selection, an inter-type cross between these two materials was performed. Subsequently, the BC_1F_1 population [(Y3 × Beinhart1000-1) × Y3] including 213 progenies was used as the mapping population, which were later used to construct an SSR-based cigar tobacco map.

Genotyping of BC_1F_1 plants. Fresh young leaves were collected from Y3, Beinhart1000-1, F_1 and individual BC_1F_1 plants. The total genomic DNA isolation and purification were conducted using a modified CTAB method as described by Tong *et al*. (2016).

During the map construction in this study, a total of 19 042 SSR primer pairs were applied, including 13 645 newly published SSRs (TM-prefix SSRs; Tong *et al*. 2016) and 5397 other batches of SSR primers (PT-prefixed SSRs; Bindler *et al*. 2007, 2011), to genotype the BC_1F_1 plants.

Polymorphism detection of all the SSRs was performed as previously described by Tong *et al*. (2016). Subsequently, the whole population was genotyped using polymorphic primers under the corresponding conditions. The amplification products were separated by electrophoresis on 5% (w/v) agarose gels stained with ethidium bromide (0.1 μg/μl); for the remnant monomorphic SSRs, single-strand conformation polymorphism (SSCP) analysis or electrophoresis on 6% (w/v) denaturing acrylamide gels stained with silver nitrate was performed for genotyping to detect allele differences.

Map construction. The mapping data for each BC_1F_1 individual were scored according to the definition of the JoinMap® Ver. 4.0 (Van Ooijen 2006). Markers and genotypes with more than 10% missing data and markers that displayed significant segregation distortion ($P < 0.01$) according to Chi-square tests were removed from the analysis. The linkage groups were determined with a logarithm of odds (LOD) threshold score ≥ 3.0, and the Kosambi mapping function was used to calculate genetic distances among loci. The constructed map was drawn using the MapChart Ver. 2.22 software (Voorrips 2002).

Comparative analysis. Comparative mapping analysis was carried out between the developed linkage map (denoted as the YB map) in this study and the previous map obtained for the Y3 × K326 intra-type cross (denoted as the YK map; Tong *et al*. 2016). A total of 318 SSR markers common to both maps were evaluated for the conservation of linkage groups and locus order arrangement. The conservation of the order of the markers was tested using a Spearman correlation test in Excel. The difference between recombination fractions of the two maps was analysed using Mantel's test for correlation between matrices (Mantel 1967). The pairwise comparison was carried out with 9999 permutations using the Ntsys Ver. 2.0 software (Rohlf 1989). In addition, a visual comparative analysis was performed against the dark tobacco reference map (denoted as the HR map; Bindler *et al*. 2011) using the comparative map tools such as comparative map viewer, and the reference map constructed for inter-type cross with

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the flue-cured and dark tobacco was also used for comparative analysis.

RESULTS AND DISCUSSION

SSR marker polymorphism. Among the 13 645 TM-prefixed SSRs, traditional genotyping analysis made 359 (2.63%) polymorphic primer pairs, generating 367 polymorphic loci, and SSCP analysis made 15 (0.11%) polymorphic primer pairs generating 15 polymorphic loci from the remnant monomorphic markers. In total, 374 (2.74%) TM-prefixed SSRs showed polymorphisms and generated 382 polymorphic loci, with an average of 1.02 alleles. While after traditional genotyping analysis, 201 (3.72%) PT-prefixed SSRs showed polymorphisms and generated 204 polymorphic loci. SSCP analysis was conducted with the remnant monomorphic markers. Subsequently, 11 (0.20%) PT-prefixed SSRs showed polymorphisms and generated 11 polymorphic loci. Taking the 5397 PT-prefixed SSRs as a whole, 212 (3.93%) primers were polymorphic, and generated 215 polymorphic loci, with an average of 1.01 alleles.

With regard to polymorphisms, the SSR primers were less efficient than other PCR-based primers (RAPD, SRAP, and ISSR). Polymorphisms were detected in as little as 3.08% of the SSR primer pairs (586/19 042) here. Such a low efficiency of generating polymorphic markers by SSR has been observed in other studies (Tong *et al*. 2012, 2016), and the main reasons for the low efficiency were the lower resolution of the approach used to separate the PCR products and the low polymorphism level between parental plants.

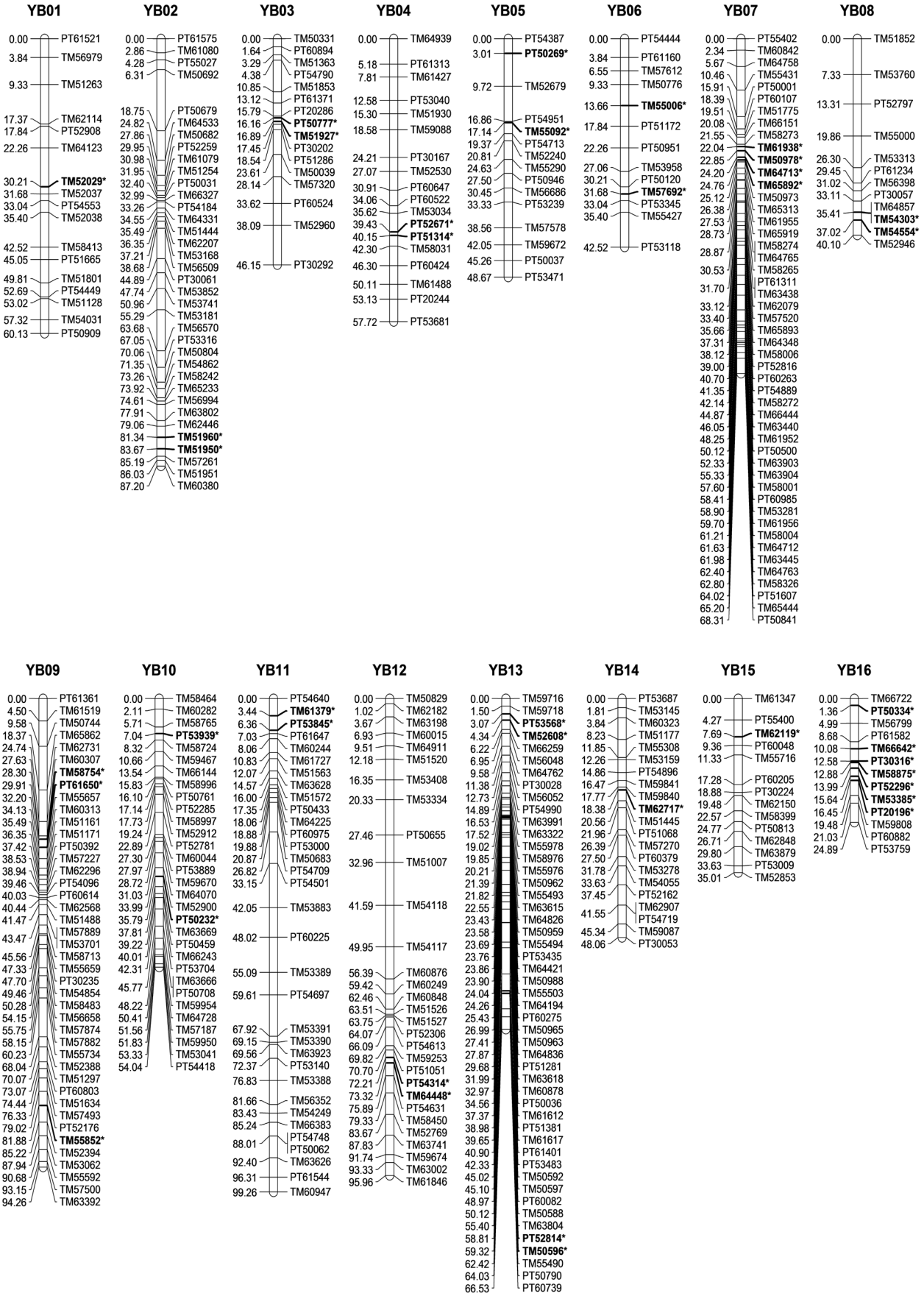
Linkage map construction. The linkage analysis data set had a total of 597 polymorphic loci that were used for map construction. After calculation, a linkage map with 562 loci distributed over 24 tentative linkage groups was constructed, and it was 1341.18 cM in total length with an average marker distance of 2.39 cM (Table 1 and Figure 1). The linkage group with the most loci was YB07 and YB13 (49 loci), whereas

Table 1. Summary of the SSR-based genetic linkage map of cigar tobacco (Y3/Beinhart1000-1)

Linkage Groups	Loci No.			Length	Min interval	Max interval	Density (cM/marker)
	total	PT[1]	TM		(cM)		
YB01	17	6 (6)	11	60.13	0.33	7.96	3.54
YB02	36	8 (6)	28	87.20	0.60	12.44	2.42
YB03	16	9 (9)	7	46.15	0.46	8.06	2.88
YB04	18	10 (9)	8	57.72	1.56	5.64	3.21
YB05	15	8 (5)	7	48.67	0.28	7.14	3.24
YB06	13	7 (6)	6	42.52	1.46	5.12	3.27
YB07	49	11 (6)	38	68.31	0.00	5.45	1.39
YB08	12	3 (3)	9	40.10	0.00	7.33	3.34
YB09	42	8 (5)	34	94.26	0.00	8.78	2.24
YB10	31	10 (6)	21	54.04	0.00	4.40	1.74
YB11	33	14 (7)	19	99.26	0.00	8.90	3.00
YB12	30	6 (5)	24	95.96	0.24	8.63	3.20
YB13	49	14 (6)	35	66.53	0.08	5.28	1.36
YB14	21	7 (6)	14	48.06	0.00	4.43	2.29
YB15	14	6 (6)	8	35.01	0.60	5.95	2.50
YB16	13	7 (5)	6	24.89	0.30	3.86	1.91
YB17	39	9 (6)	30	60.60	0.15	7.81	1.55
YB18	14	8 (8)	6	35.27	0.81	7.36	2.52
YB19	23	12 (4)	11	43.66	0.28	8.50	1.90
YB20	11	7 (7)	4	47.30	1.36	7.30	4.30
YB21	20	13 (11)	7	58.04	1.50	7.85	2.90
YB22	17	7 (4)	10	49.63	0.00	9.94	2.92
YB23	18	9 (9)	9	47.30	1.28	6.54	2.63
YB24	11	6 (6)	5	30.57	1.12	8.29	2.78
Total	562	205 (151)	357	1341.18	0.00	12.44	2.39

PT, TM – PT-/TM-series SSR primers; [1]the number in parentheses indicates the shared markers in the same linkage groups between the YB map and HR map constructed by Bindler *et al*. (2011)

https://doi.org/10.17221/121/2017-CJGPB



https://doi.org/10.17221/121/2017-CJGPB

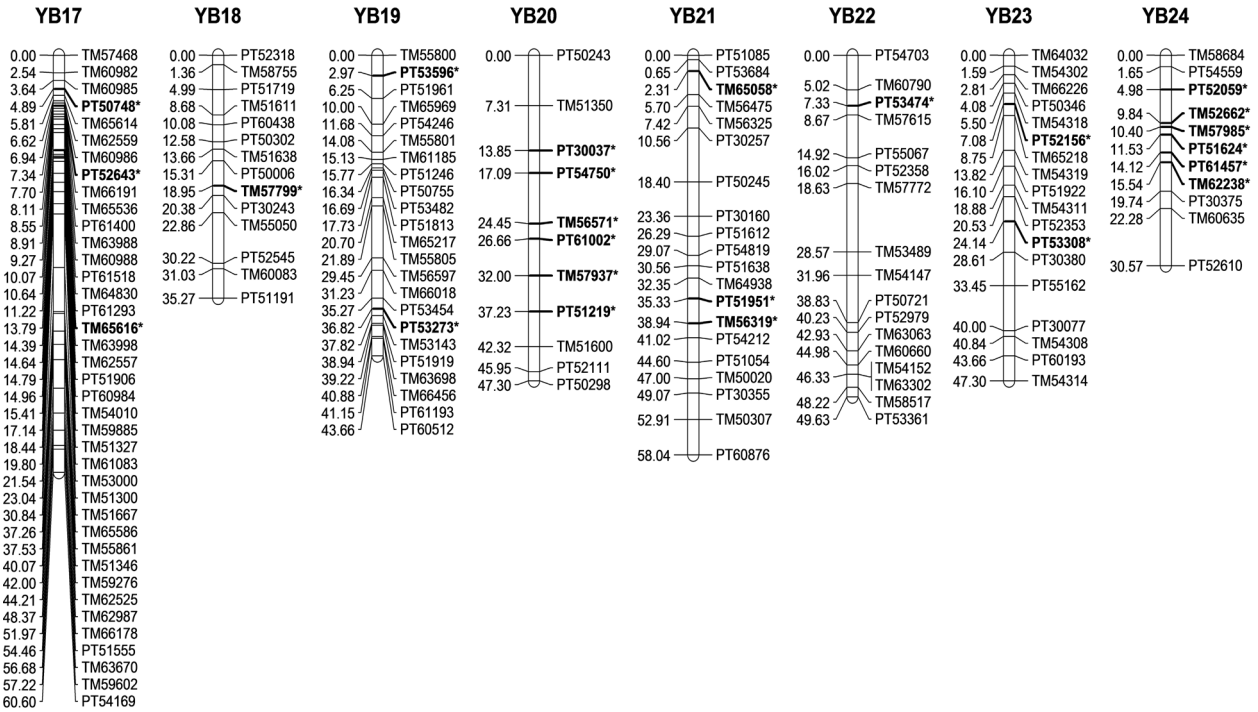


Figure 1. The genetic linkage map of cigar tobacco constructed from the BC_1F_1 population of Y3 × Beinhart1000-1; in each linkage group, the names and positions (cM) of markers are shown on the right and left side, respectively; distorted markers ($P < 0.05$) are indicated in bold and by asterisk

YB20 and YB24 had the fewest loci (11 loci), with an average locus on each linkage group of 23.4. The longest linkage group was YB11 (99.26 cM), whereas the shortest was YB16 (24.89 cM), with an average linkage group length of 55.88 cM. The largest average distance between markers was on YB01 (3.54 cM), and the shortest was on YB13 (1.36 cM). The largest gap between markers was 12.44 cM on YB02, which was also the only gap > 10 cM on the whole linkage map.

Among the 597 polymorphic loci used for map construction, 98 loci (16.42%) showed segregation distortion ($P < 0.05$), and 63 distorted loci, accounting for 11.21% of the mapped loci, were unevenly mapped on tobacco linkage groups, with 1–7 loci on each linkage group (Figure 1). The most distorted loci were on YB16 (7), YB20 (6), and YB24 (6), accounting for 30.16% of the mapped distorted loci.

Segregation distortion is a common phenomenon with all types of DNA markers. High segregation distortion has been observed in other members of the family Solanaceae, including eggplant (30%, Doğanlar *et al*. 2014), pepper (38.84%, Zhang *et al*. 2016), potato (45.13%, Bourke *et al*. 2015), and tomato (21.3%, Shirasawa *et al*. 2010). The reason for segregation distortion remains unclear, although it has been attributed to several selective factors including aberrant pollination and fertilization, the presence of lethal genes, gamete competition, abortion of gametes and zygotes, and sampling error due to a small population size (Lyttle 1991). In the current study, the use of an inter-type cross may have led to high segregation distortion, as explained by Bratteler *et al*. (2006) and Tadmor *et al*. (1987). Moreover, most markers (65%, 63/98) were skewed toward the flue-cured parent, showing an overrepresentation of Y3 alleles in our study.

Comparative analysis. The current linkage map (cigar tobacco) was compared with the HR map (dark tobacco) and previously published YK map (flue-cured tobacco) on the basis of common SSR markers (Table 2 and Figure S1 in Electronic Supplemetary Material). The Spearman correlation analysis was performed using the common SSR primer sequences of the tobacco genome on each two maps and revealed a mean correlation index of 0.93, indicating high marker order conservation. The Mantel test revealed an average correlation index of 0.81 between recombination fraction matrices. These results indicate that marker distances and orders were well conserved between each pair of maps with only slight differences.

https://doi.org/10.17221/121/2017-CJGPB

YB map *versus* HR map: One hundred fifty-two SSR markers were shared by the YB map and the HR map. The order of the SSR markers was completely conserved, except for one PT-prefixed SSR marker pair (PT51054 and PT54212 on linkage group 21). The two markers were tightly linked in both maps, but their order was reversed in our map.

YB map *versus* YK map: The common SSR markers in both maps were connected with red and green solid lines. Out of the total 318 SSR markers common to both maps, 277 (87.11%) maintained the same order, 40 (12.58%) showed order inversions and only one marker (PT54449) was mapped to a different position on linkage group 1 (approximately 55 cM in YB01, approximately 7 cM in YK01). In addition, the common SSR markers were highly conserved between the two maps, except for 20 marker pairs, which were tightly linked in both maps but their order was reversed in our study.

HR map *versus* YK map: Seventy-eight common SSR markers were distributed on all 24 linkage groups, except for linkage group 5, from which no shared SSR markers were obtained, and the numbers between the remaining 23 linkage groups ranged from 1 to 11. Out of 78 common markers, 68 (87.2%) maintained the same order, and 10 (13.8%) showed order inversions, indicating that there was a high level of collinearity between the maps.

Table 2. Statistics of the dark (HR), cigar (YB), and flue-cured (YK) tobacco comparative maps

Linkage Groups	No. of common SSR markers[1]			Variations in common marker order and position[2]		
	HR & YB	YB & YK	HR & YK	HR & YB	YB & YK	HR & YK
1	6 (1)	9 (1)	1 (1)	0	3	0
2	6 (3)	18 (3)	3 (3)	0	4	0
3	9 (3)	5 (3)	6 (3)	0	0	0
4	9 (3)	6 (3)	3 (3)	0	0	0
5	5 (0)	3 (0)	0 (0)	0	2	–[3]
6	6 (5)	6 (5)	6 (5)	0	2	2
7	7 (3)	37 (3)	3 (3)	0	4	0
8	3 (1)	4 (1)	1 (1)	0	2	0
9	5 (3)	29 (3)	3 (3)	0	0	0
10	6 (1)	19 (1)	1 (1)	0	0	0
11	7 (1)	13 (1)	1 (1)	0	0	0
12	5 (4)	21 (4)	4 (4)	0	2	0
13	6 (5)	40 (5)	5 (5)	0	2	0
14	6 (3)	13 (3)	3 (3)	0	0	0
15	6 (3)	8 (3)	3 (3)	0	4	0
16	5 (2)	4 (2)	3 (2)	0	2	2
17	6 (2)	14 (2)	2 (2)	0	2	0
18	8 (5)	7 (5)	5 (5)	0	2	2
19	4 (2)	13 (2)	2 (2)	0	2	0
20	7 (3)	5 (3)	3 (3)	0	2	2
21	11 (11)	19 (11)	11 (11)	2	2	0
22	4 (3)	9 (3)	4 (3)	0	0	0
23	9 (4)	13 (4)	4 (4)	0	4	2
24	6 (1)	3 (1)	1 (1)	0	0	0
Total	152 (72)	318 (72)	78 (72)	2	41	8

[1]HR – the map constructed by Bindler *et al.* (2011) using ***H***icks Broadleaf and ***R***ed Russian tobacco; YB – short for the genetic linkage map based on ***Y***3 and ***B***einhart1000-1 in this study; YK – the map reported by Tong *et al.* (2016) based on two flue-cured tobaccos ***Y***3 and ***K***326. The number in parentheses indicates the common markers in the three maps (HR & YB & YK); [2]indicates the number of differences in shared marker order and position between two maps; [3]indicates no shared markers in group 5 between the HR and YK maps

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In conclusion, marker orders were well conserved when comparing the present map with the other two maps, demonstrating that tobacco SSR marker positions are well established and that anchor markers can be selected for mapping studies using populations of different tobacco types for the purposes of comparative studies. However, there are some shared markers showing inconsistent orders in the three maps. Many studies have shown that minor variations of marker order and position are common among genetic maps constructed from different mapping populations (Roder *et al*. 1998; Xue *et al*. 2008). Such minor variations might not be true for chromosomes because they may result from sampling errors or other causes. However, some large variations of marker order and position, such as the large inversions in linkage groups 6, 16, 18, 20, 21 and 23 among the three maps, seem unlikely to be caused by sampling errors. Nevertheless, it is important to note that our comparative analysis was performed at the macro level, with markers spaced at millions of DNA base pairs in the genome. Thus, our analysis does not provide precise information about genomic regions related to traits of economic and quality interest or whether the QTLs detected in different populations will be present in the same regions considering different backgrounds and environmental conditions under which the crosses are performed. Furthermore, since our study established map distances, the utilization of the cigar-type tobacco can be explored in a wide range of crosses with other types of tobaccos at a lower cost and in less time.

Acknowledgments. This work was financially supported by China National Tobacco Company (2014TBB01, 110201601027(JY-01), and 110201603008) and Yunnan Tobacco Company (2014YN18, 2017YN01, and 2016YN23).

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Received for publication August 10, 2017
Accepted after corrections February 8, 2018
Published online April 23, 2018