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Original article – Thematic Issue

Association analysis of heat-resistance traits in *Clematis*

Linfang Li, Yuzhu Ma, Lulu Gao, Shu'an Wang, Peng Wang, Rutong Yang, Qing Wang and Ya Li
Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, China

Summary

The genus *Clematis* is an important ornamental vine flower popular worldwide. High temperature reduces the visual quality of *Clematis* in summer, and identification of molecular markers associated with heat resistance in *Clematis* will contribute to efficient selection of elite varieties. In our research, 127 accessions of the genus *Clematis* from nine countries were used for association analysis. These accessions were screened with 44 pairs of expressed sequence tags-simple sequence repeat (EST-SSR) primers and 22 pairs of sequence-related amplified polymorphism (SRAP) primers. A total of 133 EST-SSR and 81 SRAP polymorphic loci were detected. There were six and seven markers associated with the heat-resistance index using a General Linear Model and a Mixed Linear Model, respectively. Among these markers, SSR38-160, SSR80-230 and SRAP31-105 were significantly associated with heat resistance in both models, with effect values of 4.8–6.73%. This is the first report on association analysis of the genus *Clematis* and these markers could assist in the selection of materials for heat-resistance breeding programs and further genetic investigations.

Keywords
EST-SSR, heat-resistance index, molecular markers, queen of vines, SRAP, structure

Significance of this study

What is already known on this subject?

- *Clematis*, known as 'Queen of vines', is one of the most popular climbing plants in the world. Heat sensitivity of *Clematis* is the main problem for its cultivation in the Yangtze River region in China. Genetic research and development of molecular markers will be beneficial to solve this problem.

What are the new findings?

- In our research, 11 markers associated with the heat-resistance index were detected. Three of these markers were detected in both a General Linear Model and a Mixed Linear Model.

What is the expected impact on horticulture?

- The results indicate that heat resistance in *Clematis* is a complicated quantitative trait controlled by multiple minor genes. Our results will help in the selection of materials for heat-resistance breeding programs and further genetic investigations.

Background

The genus *Clematis*, 'Queen of vines', is one of the most popular climbing plants in the world. It has high ornamental value and is used in landscapes and floriculture as a garden or potted plant (Sheng et al., 2014; Xie et al., 2011). Most *Clematis* varieties are bred in high latitudes or cold regions and are cold resistant but heat sensitive. In our previous study, temperatures of 30–38°C in summer led to constrained growth, twig wilting, leaf wilting, shorter flowering period and abnormal flowers (Ma et al., 2016). Heat-resistance breeding has become the main direction for *Clematis* varieties in China. However, plant heat resistance is a complex quantitative trait controlled by multiple genes with complex genetic variation. Therefore, a study on the genetic and molecular basis for heat resistance in *Clematis* is necessary. Detailed study for identification of molecular markers linked to heat resistance would enhance genetic research on heat tolerance of *Clematis*.

Association mapping relies on linkage disequilibrium (LD) and is one of the most effective approaches to detect marker–trait associations in a set of samples that are not closely related (Yang et al., 2014), such as heat stress. Nine markers associated with a heat-resistance index were found in chickpea by association mapping (Thudi et al., 2014), and association analysis was conducted to identify chromosomal regions and linked markers that contribute to heat resistance. In Devasirvatham's studies, 359 DArT markers were assessed and 107 markers were linked with 11 agronomic traits expressed under heat-stressed and non-stressed conditions (Devasirvatham, 2012; Devasirvatham et al., 2016). Association analysis of heat tolerance-related traits (growth rate, turfgrass quality, survival rate, chlorophyll content and evapotranspiration rate) was conducted in 100 diverse tall fescue accessions using 1010 SSR alleles with 90 SSR markers. Finally, two SSR marker alleles were found to be associated with growth rate and evapotranspiration rate response to heat stress (Sun et al., 2015). Sequence-related amplified polymorphism (SRAP) (Li et al., 2001) and simple sequence repeat (SSR) are the molecular markers most commonly used for association mapping and genetic diversity analysis (Esposito et al., 2007; Feng et al., 2009; Fu et al., 2008; Wang et al., 2012). They have been widely used in many plant species, such as alfalfa (Ariss and Vandemark, 2007; Castonguay et al., 2010), sunflower (Fusari et al., 2012; Mandel et al., 2013) and lotus (Yang et al., 2014).

Genetic research and excellent gene-mining concerning heat resistance in *Clematis* has not been reported and there is little relevant information. It is urgent to develop an association mapping study between phenotypic variation of heat-resistance traits and gene polymorphisms of *Clematis* to enrich relevant information. In the present study, the field heat-resistance index of 127 accessions of *Clematis* were

tested in two consecutive years. Association analysis was conducted to identify expressed sequence tag (EST)-SSRs (designed from *C. chinensis* EST sequences) and SRAP markers closely associated with heat resistance in *Clematis* and to provide a theoretical basis for molecular marker-assisted breeding of new varieties with heat stress tolerance and improved heat resistance.

Materials and methods

Plant materials

In our experiment, a diverse collection of 127 accessions of *Clematis* from nine countries was selected to perform an association analysis, including 36 *Clematis* species and 91 *Clematis* varieties (Supplemental Table 1). There were 36 *Clematis* accessions collected from East China, including 14 species. The wild species were grouped according to the Flora of China and included representative species of four sections of *Clematis*. There were 91 *Clematis* varieties collected from Europe and Japan including England (31), Poland (26), Japan (15), France (5), The Netherlands (2), Sweden (2), USA (1), Argentina (1) and unknown (6). The varieties were grouped according to the International Clematis Society's latest release of the 'International Clematis Register and Checklist'. All accessions were cultivated in the Genebank of the Genus *Clematis*, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences (31°56'44.02"N, 118°48'52.18"E).

Heat-resistance index evaluation and data analysis

We systematically observed the situation of heat damage in *Clematis* in summers of 2014 and 2015. A heat-resistance index was recorded when average daily temperature exceeded 30°C for more than 3 d. The following traits were considered in calculating the heat-resistance index: leaf color and young and old leaf injury (Kang et al., 2002). The grading standard follows:

(1) *Leaf color*. Level 0: green, no heat injury symptoms (≤10%); Level 1: small part of leaf yellow (≤40%); Level 2: most of the leaves yellow (≤80%); Level 3: almost all leaves yellow (>80%).

(2) *New leaf injury symptoms*. Level 0: normal, no heat injury symptoms (≤10%); Level 1: new leaf wilting (≤40%); Level 2: new leaves withered (≤80%); Level 3: new leaves completely dried up (>80%).

(3) *Old leaves injury symptoms*. Level 0: normal, no heat injury symptoms (≤10%); Level 1: basal leaves wilted partly (≤40%); Level 2: basal leaf wilting completely (≤80%); Level 3: whole plant leaves dry up (>80%).

Observations were made from three individuals selected from each accession. Heat resistant index of the tested materials was calculated according to the thermal damage characters (above) recorded.

The heat resistant index = Σ each level per plant/(the highest level × total plants) × 100%.

Heritability (h^2) was calculated using PROCMIXED (SAS Institute, Version 9.1, Cary, NC, USA) to examine the consistency and accuracy of phenotypic data of heat-resistance index across two years, using $h^2 = \sigma g^2/(\sigma g^2 + \sigma ge^2/e + \sigma e^2/re)$. Least square means were calculated based on the pooled data from two replications because $h^2 > 0.5$ (Yu et al., 2013).

DNA isolation

Approximately 5 g of fresh leaf material was collected from each plant and immediately frozen in liquid nitrogen for DNA extraction. DNA was extracted from the leaf material by the cetyltrimethyl ammonium bromide method (Doyle and Doyle, 1990). The quantity and quality of extracted DNA were evaluated by Onedrop (OD2000, China) spectrophotometer and 1% agarose gel electrophoresis. The DNA was diluted to a concentration of 70 ng, and stored at -20°C for further use in the EST-SSR and SRAP marker analyses.

EST-SSR design and molecular marker assays

EST-SSR primers designed based on 5199 EST sequences of *C. chinensis* were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/pubmedhttp://www.ncbi.nlm. nih.gov/pubmed). Primers were designed defining loci in the range of 100–400 bp in length by software Primer Premier 5.0. For the primers, the value of the optimal melting temperature was 50–60°C, and the optimal size was 13–25 bases (Thiel et al., 2003). There were 100 SSR and 105 SRAP primers (Li and Quiros, 2001) synthesized by Shanghai Invitrogen Corporation (Shanghai, China).

Each EST-SSR primer was analyzed by polymerase chain reaction (PCR) in 10 µL of reaction mixture containing 70 ng of DNA, 1 µL of 10× buffer, 2.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs, 0.25 µmol L^{-1} of each primer and 0.5 U of Taq DNA polymerase (TaKaRa, Dalian, China). Amplification reactions were initiated with a pre-denaturing step (95°C for 3 min), followed by denaturing (95°C for 40 s), annealing (50–60°C for 40 s) and extension (72°C for 30 s) for 30 cycles, and a final elongation step at 72°C for 4 min. SRAP primer amplification system: The 10 µL of each PCR mixture comprised 70 ng of DNA, 1 µL of 1 × PCR buffer, 2.0 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs, 0.7 mmol L^{-1} of each primer and 0.5 U of Taq DNA polymerase (TaKaRa). The PCR amplification program was as follows: 5 min of denaturation at 94°C; five cycles of 45 s of denaturation at 94°C, 1 min of annealing at 35°C and 1.5 min of elongation at 72°C, then 35 of the same cycles with annealing temperature changed to 50°C; and a final elongation step of 8 min at 72°C.

Amplified PCR products were separated on 8% denaturing polyacrylamide gels using a vertical electrophoresis device. Detection of EST-SSR and SRAP bands were visualized by the silver staining method (Eujayl et al., 2004).

The polymorphic information content (PIC) was estimated as follows:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where PIC_i is the PIC of marker i, P_{ij} indicates the frequency of the j^{th} allelic variation for locus i, and the bands of marker i are from 1 to n.

Population structure analysis and association mapping

The structure of the 127 *Clematis* accessions was assessed by STRUCTURE 2.3.4 software (Evanno et al., 2005). with 44 EST-SSR and 22 SRAP markers. The optimal number of subpopulations (K) was inferred from 1 to 10, with five runs at each K. For each run, 10,000 burn-ins were performed followed by 100,000 Markov chain Monte Carlo (MCMC) simulations. The maximum likelihood was determined by the log-likelihood of the data Ln P(D) in the STRUCTURE output or ΔK, with ΔK based on the change in Ln P(D) between successive K values. $\Delta K = m(|L(K + 1) - 2L(K) + L(K - 1)|)/s[L(K)]$, $L(K)$ is Ln P(D) (Evanno et al., 2005).

Molecular marker–phenotypic trait associations were identified using GLM and MLM in TASSEL2.1 (Bradbury et al., 2007). The Q-value identified by running STRUCTURE at

Supplemental Information – Table S1. *Clematis* accessions investigated in this study.

No.	Name	Group	Origin	No.	Name	Group	Origin
1	*C.* 'Patricia Ann Fretwell'	Early large-flowered	England	49	*C.* 'Freckles'	Cirrhosa	Japan
2	*C.* 'The Vagabond'	Early large-flowered	England	50	*C.* 'Flore-pleno'		Japan
3	*C.* 'Vyvyan Pennell'	Early large-flowered	England	51	*C.* 'Blue Pillar'	Early large-flowered	Japan
4	*C.* 'Andromeda'	Early large-flowered	England	52	*C. cartmanii* 'Joe'	Forsteri	Japan
5	*C.* 'Gillian Blades'	Early large-flowered	England	53	*C.* 'Bagatelle'	Late large-flowered	Japan
6	*C.* 'Violet Elizabeth'	Early large-flowered	England	54	*C.* 'Sylvia Denny'	Early large-flowered	Japan
7	*C.* 'Maidwell Hall'	Atragene	England	55	*C.* 'Laura'	Late large-flowered	Poland
8	*C.* 'Markham's Pink'	Atragene	England	56	*C.* 'Sympatia'	Late large-flowered	Poland
9	*C.* 'Chalcedony'	Early large-flowered	England	57	*C.* 'Vistula'	Late large-flowered	Poland
10	*C.* 'Bees Jubilee'	Early large-flowered	England	58	*C.* 'Wildfire'	Early large-flowered	Poland
11	*C.* 'Beauty of Worcester'	Early large-flowered	England	59	*C.* 'Danuta'		Poland
12	*C.* 'Hagley Hybrid'	Late large-flowered	England	60	*C.* 'Barbara'	Early large-flowered	Poland
13	*C.* 'Kiri Te Kanawa'	Early large-flowered	England	61	*C.* 'Diamond Ball'	Early large-flowered	Poland
14	*C.* 'Princess Diana'	Texensis	England	62	*C.* 'Westerplatte'	Early large-flowered	Poland
15	*C.* 'Arabella'	Integrifolia	England	63	*C.* 'Kardynal Wyszyńsk'	Late large-flowered	Poland
16	*C.* 'Jenny Caddick'	Viticella	England	64	*C.* 'Sunset'	Early large-flowered	America
17	*C.* 'Elf '	Viticella	England	65	*C.* 'Doctor Ruppel'	Early large-flowered	Argentina
18	*C.* 'Countess of Lovelace'	Early large-flowered	England	66	*C.* 'Polish Spirit'	Late large-flowered	Poland
19	*C.* 'Mrs Cholmondeley'	Early large-flowered	England	67	*C.* 'Solidarnosc'	Early large-flowered	Poland
20	*C.* 'Veronica's Choice'	Early large-flowered	England	68	*C.* 'Monika'	Atragene	Poland
21	*C.* 'Proteus'	Early large-flowered	England	69	*C.* 'Niobe'	Early large-flowered	Poland
22	*C.* 'Jan Pawel II''	Early large-flowered	England	70	*C.* 'Królowa Jadwiga'	Early large-flowered	Poland
23	*C.* 'Royalty'	Early large-flowered	England	71	*C.* 'Ruutel'	Early large-flowered	Poland
24	*C.* 'Little Mermaid'	Early large-flowered	England	72	*C.* 'Kardynal Wyszyński'	Late large-flowered	Poland
25	*C.* 'Louise Rowe'	Early large-flowered	England	73	*C.* 'Lech Wałęsa'		Poland
26	*C.* 'Isago'	Early large-flowered	England	74	*C.* 'Kacper'	Early large-flowered	Poland
27	*C.* 'Duchess of Edinburgh'	Early large-flowered	England	75	*C.* 'Regina'	Early large-flowered	Poland
28	*C.* 'Jackmanii'	Late large-flowered	England	76	*C.* 'Hania'		Poland
29	*C.* 'Shangdiyi'		England	77	*C.* 'Ashva'		Poland
30	*C.* 'Snowdrift'	Armandii	England	78	*C.* 'Krakowiak'	Viticella	Poland
31	*C.* 'Romantika'	Late large-flowered	Estonia	79	*C.* 'Ernest Markham'	Late large-flowered	Poland
32	*C.* 'Viola'	Late large-flowered	Estonia	80	*C.* 'Empress'	Early large-flowered	Poland
33	*C.* 'Comtesse de Bouchaud'	Late large-flowered	France	81	*C.* 'Mazowsze'	Late large-flowered	Poland
34	*C.* 'Etoile Violette'	Viticella	France	82	*C.* 'Ooh La La'		Poland
35	*C.* 'Nelly Moser'	Early large-flowered	France	83	*C.* 'Janny'	Atragene	
36	*C.* 'Ville de Lyon'	Late large-flowered	France	84	*C.* 'Ivan Olsson'	Early large-flowered	Sweden
37	*C.* 'Frau Susanne'	Early large-flowered	France	85	*C.* 'Thyrislund'	Early large-flowered	Sweden
38	*C.* 'Blue Light'	Early large-flowered	Holland	86	*C.* 'Xerxex'	Early large-flowered	Unknown
39	*C.* 'Multi Blue'	Early large-flowered	Holland	87	*C.* 'Justa'	Viticella	Unknown
40	*C.* 'Rooran'	Early large-flowered	Japan	88	*C.* 'Betty Risdon'	Early large-flowered	Unknown
41	*C.* 'Crystal Fountain'	Early large-flowered	Japan	89	*C.* 'Moonlight'	Early large-flowered	Unknown
42	*C.* 'Omoshiro'	Early large-flowered	Japan	90	*C.* 'Apple Blossom'	Armandii	Unknown
43	*C.* 'Kakio'	Early large-flowered	Japan	91	*C. patens* -1	Sect. Viticella DC.	Unknown
44	*C.* 'Hakuookan'	Early large-flowered	Japan	92	*C. patens* -2	Sect. Viticella DC.	China
45	*C.* 'Shin-shigyoku'	Early large-flowered	Japan	93	*C. patens* -3	Sect. Viticella DC.	China
46	*C.* 'Kaiser'	Early large-flowered	Japan	94	*C. patens* -4	Sect. Viticella DC.	China
47	*C.* 'Asao'	Early large-flowered	Japan	95	*C. florida*.var. *Plena*	Sect. Viticella DC.	China
48	*C.* 'Rooguchi'	Integrifolia	Japan	96	*C. lanuginosa*	Sect. Viticella DC.	China

Table S1. Continued.

No.	Name	Group	Origin	No.	Name	Group	Origin
97	*C. lanuginose -1*	Sect. Viticella DC.	China	113	B1		China
98	*C. cadmia*	Sect. Viticella DC.	China	114	B2		China
99	*C. finetiana -1*	Sect. Clematis	China	115	B3		China
100	*C. finetiana -2*	Sect. Clematis	China	116	B4		China
101	*C. chinensis* -1	Sect. Clematis	China	117	B5		China
102	*C.chinensis* -2	Sect. Clematis	China	118	B6		China
103	*C. chinensis* -3	Sect. Clematis	China	119	B7		China
104	*C. henryi* -1	Sect. Viorna (Reichb.) Prantl	China	120	B8		China
105	*C. henryi* -2	Sect. Viorna (Reichb.) Prantl	China	121	B9		China
106	*C. heracleifolia*	Sect. Viorna (Reichb.) Prantl	China	122	B10		China
107	*C. tangutica*	Sect. Meclatis (Spach) Tamura	China	123	B11		China
108	*C. hexapetala*	Sect. Clematis	China	124	B12		China
109	*C. uncinata*	Sect. Clematis	China	125	B13		China
110	*C. lasiandra*	Sect. Viorna (Reichb.) Prantl	China	126	B14		China
111	*C. acerifolia*	Sect. Cheiropsis DC.	China	127	B15		China
112	*C. apiifolia*	Sect. Clematis	China				

K = 2 was used to define the membership of the model based populations. To estimate the genetic relatedness among inbred lines, the K-value (kinship matrix) was constructed on the basis markers using the software SPAGeDi (Hardy and Vekemans, 2002). Significance of a marker–trait association was set at $P < 0.01$, with the relative magnitude represented by the R^2 value as the proportion of variation explained by the marker (Yang et al., 2014).

Results

Phenotype evaluation

The phenotype h^2 was 0.51 between the two environments in 2014 and 2015 (Supplemental Table S2), indicating high repeatability over tested environments. Thus, least square means of individual traits were calculated across all environments tested for association analysis. The heat-resistance index was in the range of 0–25.5% (Table 1). The average of phenotype was 9.42% and the coefficient of variation was 77.38% (Table 1). These results suggest that heat-resistant properties had good diversity and were suitable for association analysis.

Molecular polymorphism

There were 100 EST-SSR and 106 SRAP markers used for selection of polymorphism. There were 44 EST-SSR (Supplemental Table 3) and 22 SRAP markers (Supplemental Table 4) with high polymorphism selected for population polymorphism detection. The 44 SSR markers generated a total of 133 polymorphic alleles among the 127 accessions (Table 2). The average number of alleles per EST-SSR primer was 3.02, with numbers in the range of 3–4 (Table 2 and Supplemental Table 4). The PIC of EST-SSR markers was in the range of 0.19–0.83 (Table 2 and Supplemental Table 4). The 22 SRAP markers generated 81 polymorphic alleles among the population. The number of polymorphic fragments for each primer was in the range of 3–6 (Table 2 and Supplemental Table 4), with mean of 3.68. The PIC of SRAP markers was in the range of 0.12–0.62 (Table 2 and Supplemental Table 4).

Supplemental Information – Table S2. Heritability of heat-resistance index in *Clematis*.

	Heritability
Heat-resistance index	0.51

Population structure

The population was clustered into two major subpopulations according to structure analysis (Figure 1): C1 and C2 (Figure 2). Using a membership probability threshold of 0.50, 26 accessions were assigned to C1 and 101 to C2 (Figure 2). C1 was a diverse group including 36.11% *Clematis* accessions from East China. These accessions were group of atragene (2), *C. viticella* (1), early large-flowered (5), late large-flowered (1), *C. integrifolia* (1), unknown (6), Sect. *Clematis* (5), Sect. *Viticella* DC. (4), and Sect. *Viorna* (Reichb.) Prantl (1). C2 was the largest group, with 101 accessions of mixed origins, including approximately 86.67% of cultivated accessions and some accessions of *Clematis* accessions from China (Figure 2 and Supplemental Table 1).

Association mapping

In the present study, GLM and MLM models were used for association mapping. Heat-resistance index and 214 genetic loci from 44 EST-SSR and 22 SRAP markers were used for association mapping. The GLM model showed that six markers (SRAP23-175, SRAP25-240, SRAP31-105, SSR38-160, SSR80-230 and SSR31-245; $P < 0.01$) were significantly associated with the heat-resistance index and explained about 5.20–8.96% of the phenotypic variation (Table 3). The most significantly associated EST-SSR marker (P = 6.96E-04) was SSR80-230 and explained the highest amount (8.96%) of the phenotypic variation (Table 3).

In the MLM model, seven markers (SS38-160, SSR80-230, SSR96-130, SRAP23-150, SRAP31-105, SRAP24-170 and SRAP24-220) had significant association with the heat-resistance trait, and explained about 38.26% of the total phenotypic variation (Table 3). Among these, the most significantly associated EST-SSR marker was SSR38-160, which explained

Table 1. Heat-resistance index of *Clematis* accessions.

Trait	Max.	Min.	Mean	R	SD	CV
Heat-resistance index	25.5%	0	9.42%	25.5%	7.29%	77.38 %

Supplemental Information – Table S3. Sequence of EST-derived SSRs for *C. chinensis* used for association mapping.

Marker	Forward primer	Reverse primer
SSR1	AGAGGTGAGAATGGGAGC	AGAGGGGCAAGATTTATT
SSR2	AGAGGACGATTCTACCGC	GTCGATCTCAGCCAGTTT
SSR5	AAACACCTTTCCTCCGCCTCTTA	AAGATGGGTAATCCCTCGTTTGTG
SSR8	CCCCTTCCTTTGATTTCTG	TGCGATGTTCTCGTTTTCT
SSR15	AGAACTGAGAGCGAAGA	TAGGAGACAGGCGAAA
SSR20	GAAGGAAAATCTCCGTAAAGC	AAGACCGCAAACCAACACC
SSR26	ACGCCAACAGTTCCCCCTC	CGCAACCGTCACCCCGA
SSR31	GGCTCCAACCTCTG	ACCGCCTCTACCAC
SSR34	AGTGGCAGTGGGAGTGGTG	GGAGGTGGCATTCGGTAGA
SSR35	TTTCAACAATACCCTA	TCCTCACCTTCCTAC
SSR36	ACGGGAGCACTGAT	TGACCGAAGCAACT
SSR37	CAGCAATGGATGGC	GCACGGGAAAGAAA
SSR38	TCAATCTCTCTCCCCA	ACCAAGTGTTCTACCCA
SSR39	CAAGAAAATCACCAAAA	TGACTAAGATGAGCCAA
SSR49	CTCATCGCTCCTCTGTTC	CGGTTTTCCATTATTCAA
SSR51	CACCAAAAACGGTTACAA	CATCCCAGAGACGAGAAT
SSR53	CTCTTTCCCTCAG	CAACCTTTCAATC
SSR56	TCTACAAAGAGCACACGC	GAACGCCCCGATAACT
SSR57	CTCTCTCCTGCTCTTGT	AACTTTGGATTTTGATG
SSR58	CCCCAAACCCCACACTG	CCCCACGCACCCATAGA
SSR59	AGAAGAACAGAAAGCAGAA	CGATGAGATTACCAGTGAC
SSR62	AGACTGATTTCGTTGCG	TGAGTGCTTGGGGGA
SSR63	TTAGCAGTGGCAGTGGG	CAGAGGTGAGGGGGTTT
SSR66	CTTCTCCGCTTCCTCCA	CATCGCCATCCATTGCT
SSR68	CAAAACTACCCTCCTC	CTGACTCTGACCCCTA
SSR69	CCCGCTCATTCTGTTATC	AGGGTTTCTCCGTCGTT
SSR70	TTCATCTATGCCCAG	GAAAGCAAGAACCCT
SSR72	TCCCACTCCACAATCTCTCTG	AATCACTTGGCACACTTCCC
SSR74	ATCCATTTTCATTCTTCGG	TAGTTTCCACCTTCACCAG
SSR76	AAGGTTGTGTCGGTTAC	GAGTTGAGTCGGATTTG
SSR78	TTACGACGAACCACA	AACCGCTAACCCTT
SSR80	ACAAGAGAAAACCCGAAA	GCAGTCCCAGCCGAG
SSR82	CTCCCTCCTCCTCAAA	AGCCATCACCTCATACA
SSR84	TACCACAAGCAAGATG	AGGAAGAAAGTGAAAAC
SSR85	CTACTTCTTCTTCGCC	TATCTGCCTCCTCTCA
SSR86	TCCGACCCCAGTTCAC	TTCTCCTTTCTCCCGC
SSR90	TCCACCGCCTAAGACC	TCCACCGCCTAAGACC
SSR91	AAAAGCGGTGGTTATC	CTTCAGCCCTCAGTAGA
SSR92	TCTTCCTTTTTTCTCAA	AAATAATCAACCCCAT
SSR94	GCAAGATGGGAGGAA	CGAAATCGGAGGAGA
SSR95	CCCGCCAGACCAAAGT	GTGGAGAGCCGAACCC
SSR96	GCTACCGTTACAGTCC	CCTCAGTCACCATCTC
SSR97	GAAACTTATCCCCAAT	TGAAAGAGCACCCAT
SSR98	ATCACTCCACTGCTA	CTCACCCTTTATTCT

Supplemental Information – Table S4. Diversity statistics of SSR and SRAP markers used for association mapping.

Marker	NP[a]	PIC[b]	Marker	NP[a]	PIC[b]	Marker	PC[c]	NP[a]	PIC[c]
SSR1	4	0.40	SSR63	3	0.72	SRAP1	Me2 Em11	3	0.33
SSR2	3	0.60	SSR66	3	0.77	SRAP4	Me1 Em16	3	0.5
SSR5	3	0.28	SSR68	3	0.57	SRAP8	Me11 Em16	4	0.22
SSR8	3	0.52	SSR69	3	0.37	SRAP14	Me10 Em5	3	0.12
SSR15	3	0.61	SSR70	3	0.66	SRAP15	Me9 Em3	4	0.24
SSR20	3	0.44	SSR72	3	0.53	SRAP16	Me9 Em2	4	0.12
SSR26	3	0.38	SSR74	3	0.83	SRAP19	Me8 Em15	5	0.16
SSR31	3	0.28	SSR76	3	0.8	SRAP21	Me8 Em13	6	0.19
SSR34	3	0.19	SSR78	3	0.72	SRAP22	Me8 Em11	4	0.33
SSR35	3	0.28	SSR80	3	0.61	SRAP23	Me7 Em7	3	0.39
SSR36	3	0.4	SSR82	3	0.55	SRAP24	Me7 Em6	3	0.47
SSR37	3	0.67	SSR84	3	0.49	SRAP25	Me7 Em5	4	0.55
SSR38	3	0.83	SSR85	3	0.44	SRAP26	Me7 Em4	5	0.21
SSR39	3	0.51	SSR86	3	0.42	SRAP27	Me7 Em3	3	0.49
SSR49	3	0.31	SSR90	3	0.45	SRAP28	Me7 Em2	4	0.32
SSR51	3	0.34	SSR91	3	0.38	SRAP29	Me7 Em1	3	0.62
SSR53	3	0.73	SSR92	3	0.65	SRAP30	Me6 Em18	4	0.24
SSR56	3	0.32	SSR93	3	0.69	SRAP31	Me6 Em16	4	0.51
SSR57	3	0.54	SSR95	3	0.67	SRAP32	Me6 Em15	3	0.22
SSR58	3	0.37	SSR96	3	0.27	SRAP33	Me6 Em13	3	0.39
SSR59	3	0.21	SSR97	3	0.71	SRAP41	Me4 Em15	3	0.24
SSR62	3	0.55	SSR98	3	0.73	SRAP42	Me1 Em15	3	0.62

[a] Number of polymorphic bands per primer.
[b] PIC is polymorphic information content.
[c] SRAP primer combinations.

Table 2. Polymorphism information for molecular markers.

Molecular marker	NPP[a]	NPL[b]	NAP[c]	PIC (range)[d]
EST-SSR	44	133	3.02 (3–4)	0.522 (0.19–0.83)
SRAP	22	81	3.68 (3–6)	0.342 (0.12–0.62)
Total	66	214		

[a] Number of primer pairs.
[b] Number of polymorphic loci.
[c] Number of alleles per primer mean (range).
[d] PIC is polymorphism information content.

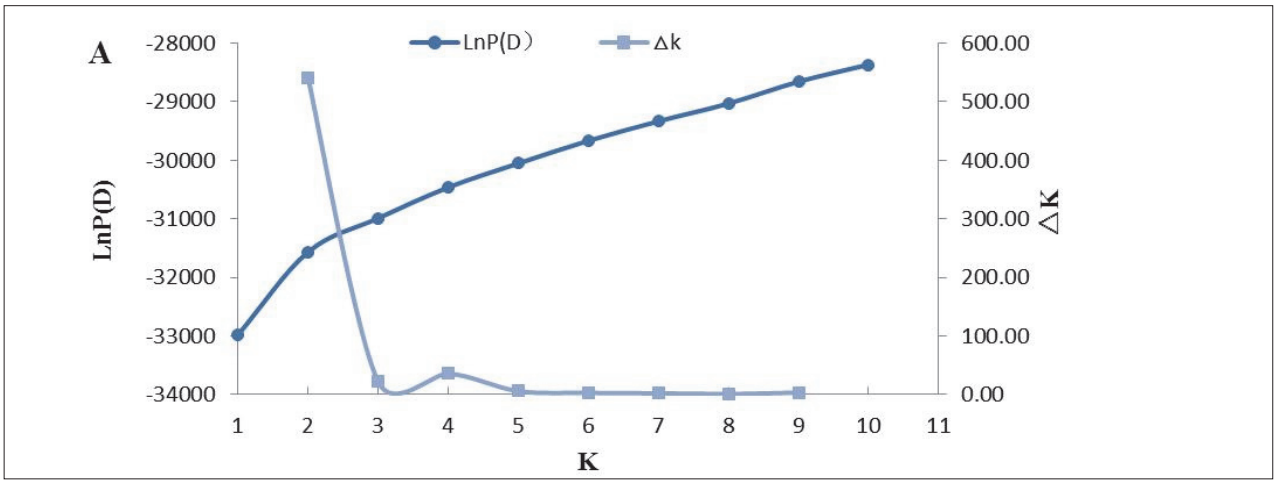


Figure 1. Analysis of *Clematis* population structure. Estimated [Ln P(D)] and ΔK of STRUCTURE analysis of all accessions based on molecular marker.

about 5.29% of the total phenotypic variation. SRAP23-150 explained the highest amount of phenotypic variation with 10.56%. A total of 11 loci were detected under the two models. SSR38-160, SSR80-230 and SRAP31-105 were detected in both the GLM and MLM models and explained 6.73, 8.96 and 5.20% of phenotypic variation for GLM, respectively, and correspondingly 5.29, 5.47 and 4.80% for MLM (Table 3).

Discussion

With global climate warming, research on heat resistance of plants has become a hot topic and strong heat-resistant varieties have become breeding goals for many economic plants (De Souza et al., 2012; Devasirvatham, 2016; Lavania et al., 2015). The genus *Clematis* includes excellent ornamental vine plants with potential application in the middle and lower reaches of the Yangtze River in China. It is urgent to breed strong heat-resistant varieties and to research heat resistance. We evaluated the heat resistance among *Clematis* accessions and identified molecular markers associated with heat resistance to lay a theoretical foundation for cultivation of new heat-resistant varieties by molecular marker-assisted breeding.

The genus *Clematis* contains more than 300 species and more than 2000 varieties comprising a diverse mix of plant and flower form, size and color, and multiple stress resistance (Yanfei et al., 2009). After nearly 200 years of cultivation, most varieties are highly heterozygous and their parents are unknown. There were 127 *Clematis* accessions used in our research, which were mainly subdivided into two major subpopulations: 86.67% cultivated accessions were grouped in C2, with some accessions from East China including *C. lanuginosa* and *C. heracleifolia* (common hybrid parents) also grouped in C2. *Clematis hexapetala* and *Clematis* 'Barbara' were the most distant accessions with genetic and morphological differences. Other accessions were more or less gene permeable. This also showed that *Clematis* varieties derived from hybridization of multiple *Clematis* species and showed a high degree of heterozygosity (Figure 2).

The method of identifying heat resistance can be divided into early and later identification according to seedling age. The early identification method includes seed germination stage identification (Egley, 1990) and artificially simulating the environment for the determination of seedling stage plants (Yin, 2008; Zhang, 2015). The later identification is

Table 3. Significant associations for heat-resistance index estimated with GLM and MLM models for *Clematis* accessions ($P < 0.01$).

Trait	GLM			MLM		
	Locus [c]	P-value[b]	R^2 (%)[a]	Locus	P-value[b]	R^2 (%)[a]
Heat-resistance index	SSR80-230	6.96E-04	8.96	SSR38-160	2.39E-04	5.29
	SSR31-245	4.10E-03	6.7	SSR80-230	7.42E-04	5.47
	SSR38-160	4.51E-03	6.73	SSR96-130	4.30E-03	2.79
	SRAP23-175	4.34E-03	6.55	SRAP23-150	1.96E-06	10.56
	SRAP25-240	5.62E-03	7.33	SRAP31-105	2.25E-03	4.8
	SRAP31-105	8.26E-03	5.2	SRAP24-170	4.31E-03	3.77
				SRAP24-220	6.72E-03	5.58

[a] R^2 value indicates the percentage of phenotypic variation explained by the marker.
[b] indicates the significance between marker and the phenotype.
[c] Marker code – fragment length.

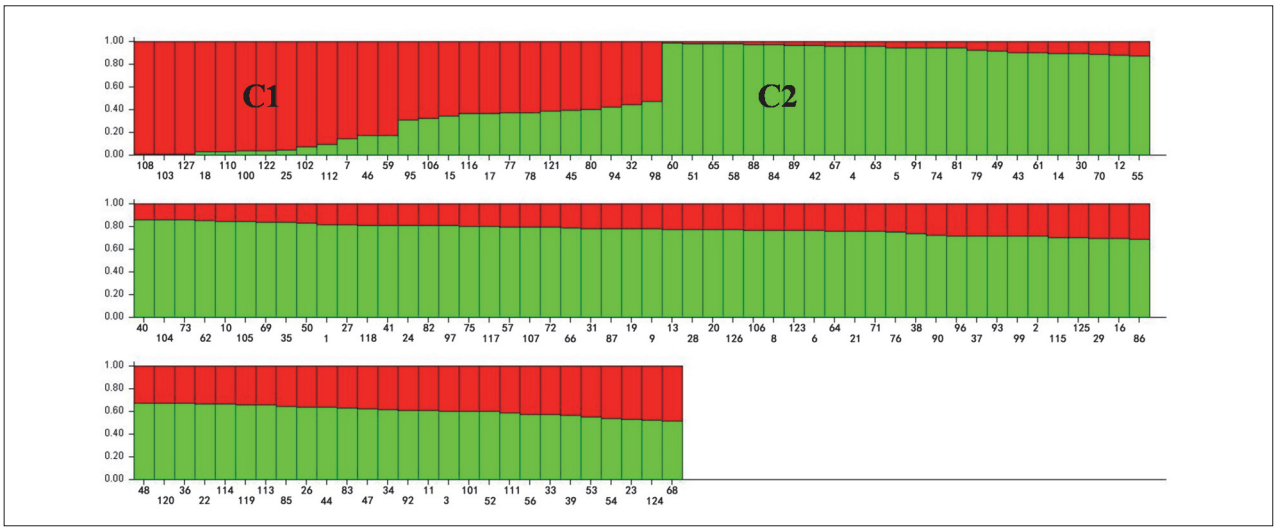


Figure 2. Genetic relatedness of 123 *Clematis* accessions with 44 SSRs and 22 SRAPs as analyzed by the STRUCTURE program. Numbers on the y-axis indicate the membership coefficient. The color of the bar indicates the two groups identified through the STRUCTURE program (C1 = red, C2 = green). Accessions with the same color belong to the same group.

basically performed in field experiments by observing field characteristics of plants under natural high-temperature conditions (Yin, 2008). The *Clematis* seedling age for our study was 5–6 years, and so was more suitable for the field observation method. This method used to identify heat resistance is widely used with advantages of objectivity, accuracy (Kang et al., 2002; Si-jian, 2005). Through our preliminary study, we used the least square means of two years of data to improve the accuracy of the results when phenotype $h^2 > 0.50$ (Yu et al., 2013). There was diversity of heat resistance in these varieties and accessions, making them suitable for association analysis.

In *Clematis*, Gardner et al. (2005) used four ISSR markers to fingerprint 32 vining varieties and five non-vining species (*C. fruiticosa*, *C. heracleifolia*, *C. integrifolia*, *C. hexapetala*, and *C. recta*), which showed rich genetic diversity. In our study, we used SSR and SRAP markers to analyze the population structure of 127 accessions. The average number of alleles in SSR and SRAP markers was 3.02 and 3.68, respectively. There were 214 polymorphic loci detected by 44 EST-SSR and 22 SRAP markers (Table 2) and they showed good polymorphism in the population.

In association mapping in plants, the stratification of subpopulations intensifies LD. In other words, population stratification and unequal distribution of alleles can result in false-positive associations (Flint-Garcia et al., 2003). To overcome this problem, two population structure scenarios were considered and the results stable across the two scenarios were reported. The population structure was suitable for association analysis. Achleitner et al. (2008) stated that different results may illustrate the relative importance of different parts of the population structure accounted for by different models. Theoretically, accuracy should be higher when markers are detected using two or more methods. Chitwood et al. (2016) used single marker regression (SMR), GLM and MLM in TASSEL to analyze the association of markers with bolting, plant height and erectness in spinach (*Spinacia oleracea* L.). GLM was use for association analysis of five traits (pod fiber, seeds per pod, plant type, growth habit and days to flowering) in diverse common bean accessions (Nemli et al., 2014). Three models (SMR, GLM and MLM) were used to analyze the association of markers with cowpea bacterial blight resistance in cowpea (*Vigna unguiculata*) (Shi et al., 2016). In the present study, GLM and MLM models were used for association mapping, and 10 markers were detected as associated with heat resistance. SSR80-230 and SRAP23-150 were the markers most strongly associated with the heat-resistance index in the GLM and MLM models (Table 3). SRAP23-150 was the most contributing locus, with phenotypic variation of 10.56%. The markers SSR38-160, SSR80-230 and SRAP31-105 were detected by both models. These markers were the major locus in this study and the low false-positive probability made this suitable for selection of materials for heat-resistance breeding programs and other genetic investigations.

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Addresses of authors:
Linfang Li[1], Yuzhu Ma[1], Lulu Gao, Shu'an Wang, Peng Wang, Rutong Yang, Qing Wang and Ya Li*
Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing 210014, China
[1] Linfang Li and Yuzhu Ma have contributed equally to this work.
* Corresponding author; E-mail: yalicnbg@163.com