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Full Length Article

A Genetic Linkage Map of *Phalaenopsis*-based on AFLP Markers and the "Two-way Pseudo-testcross" Mapping Strategy

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Abstract

Phalaenopsis are grown widely as cut-flowers and potted plants in the floriculture trade. Here, we report the genetic linkage maps for *Phalaenopsis*, which were developed by genotyping 88 F_1 progenies from a cross between *Phalaenopsis* '462' (male) and *Phalaenopsis* '20' (female) with amplification fragment length polymorphisms (AFLP) in a "two-way pseudo-testcross" mapping strategy. For a total of 416 polymorphic loci showing Mendelian segregation were generated from 71 AFLP primer combinations. The data were analyzed using JoinMap 3.0 to construct the parent-specific linkage maps. The paternal map consisted of 15 linkage groups and 122 AFLP makers, covering 820.3 cM with a mean distance of 6.7 cM between adjacent markers. The maternal map consisted of 14 linkage groups with 175 loci, resulting in a total genetic distance of 878.3 cM and an average genetic distance of 5.0 cM between adjacent markers. Yet, as far as we know, this is the first study to construct the linkage map of *Phalaenopsis*. The two maps will serve as the frameworks for mapping of horticultural quantitative trait locis (QTLs) and furnish reference information for the application of future marker-assisted selection in *Phalaenopsis* breeding. © 2017 Friends Science Publishers

Keywords: Orchid; Molecular marker; Genetic analysis; Linkage

Introduction

The genus *Phalaenopsis* is one of the most commercially important orchids in the family Orchidaceae, grown as cut flowers and pot plants due to their long-lasting flowers and graceful appearance. *Phalaenopsis* industry is growing around the world in recent decade. Numerous varieties of *Phalaenopsis* have been developed and registered with the Royal Horticulture Society (RHS) each year. However, very limited research efforts have been devoted to understanding the genetic diversity (Been *et al.*, 2002; Chang *et al.*, 2009) or the genomic features of *Phalaenopsis* (Hsu *et al.*, 2008) and genetic transformation (Liao *et al.*, 2004; Sjahril *et al.*, 2006). Although one *Phalaenopsis* genome was recently sequenced (Cai *et al.*, 2015), the inheritance of important ornamental or horticultural traits, genetic relationships among important species, and the genomic organization of *Phalaenopsis* remained poorly understood, due to the complex genetic background and extensive hybridization in this genus.

Genetic linkage maps provide a powerful tool for comprehending the genetic basis of sophisticated traits in many plants (Lee, 1995). Particularly they are very useful for map-based cloning and the localization of important genes and assisting plant breeders in the selection of breeding parents and breeding lines. Genetic mapping in plants are often constructed utilizing segregating populations from crosses between inbred lines, but this strategy is not possible for many of those plants that have high heterozygosity and long life cycles, such as forest trees (Grattapaglia and Sederoff, 1994), fruit trees (Kenis and Keulemans, 2005), perennial flowers (Dunemann *et al.*, 1999; Hibrand-Saint Oyant *et al.*, 2008), etc. For these plants, the strategy of "two-way pseudo-testcross" has been adopted frequently. With this strategy, genetic linkage maps are constructed utilizing F_1 offspring derived from the cross of two highly heterozygous individuals (Grattapaglia and Sederoff, 1994). For example, the method has been utilized successfully to construct linkage maps in agronomic crops such as peanut (Hong *et al.*, 2008) and sweetpotato (Kriegner *et al.*, 2003), ornamental plants such as wintersweet (Chen and Chen, 2010) and dendrobium (Xue *et al.*, 2010), and tree species such as pistacia (Turkeli and Kafkas, 2013) and *Eucommia ulmoides* (Wang *et al.*, 2014).

Many species in *Phalaenopsis* are diploids with a chromosome number of $2n = 2x = 38$, although some species have different chromosome numbers as revealed by cytological studies (Christenson, 2001; Kao *et al.*, 2001). Hybridization occurs easily in *Phalaenopsis,* not only between the species but also with members of related genera. Because of frequent hybridizations, most *Phalaenopsis* varieties are highly heterozygous. In addition,

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developing inbred lines in *Phalaenopsis* has been rather difficult. Considering these factors, the "two-way pseudo-testcross" strategy seemed to be an effective and convenient way for constructing genetic linkage maps in *Phalaenopsis*. The objective of this study was to apply the AFLP marker system to *Phalaenopsis*, to develop maternal and paternal pure-coupling phase AFLP markers, and construct linkage maps using the mapping strategy of "two-way pseudo-testcross". We expect that these markers and the linkage maps would be valuable for identifying markers associated with important traits of interest and improving the breeding efficiency in this important orchid.

Materials and Methods

Plant Materials

The mapping population used was a hybrid population developed from two cultivars, *Phalaenopsis* '20' and '462'. *Phalaenopsis* '20' was characterized by creamy yellow flowers with small mahogany spots, medium-sized leaves, and slight fragrance, and it was used as the female parent of the population. The male parent was *Phalaenopsis* '462', whose flowers were creamy white overlaid with large waxy magenta spots. This variety had large leaves and no fragrance. Hybrid seedlings were produced by germinating the seeds on an aseptic MS medium with the addition of sucrose at 30 $g \cdot L^{-1}$ and agar at 6 $g \cdot L^{-1}$. Seedlings were transplanted individually to plastic containers filled with sphagnum moss and then grown in an environment-controlled greenhouse for 6 months. After that 88 individual plants were randomly picked out of 450 progenies to construct the mapping population.

DNA Extraction

Genomic DNA of *Phalaenopsis* was extracted from the fresh leaves using the cetyltrimethy lammonium bromide (CTAB) methods described by Murray and Thompson (1980) with minor modifications. A 0.5 g fresh leaf tissue was pulverized using liquid nitrogen, and then the powder was gently dispersed in an extraction buffer based on Murray and Thompson (1980), and then incubated at 65°C for 20 min. During the incubation, samples were shaken gently. The extract was mixed with an equal volume of octanol:chloroform (1:24), the aqueous phase was transferred to a clean centrifuge tube after centrifugation (13,000 × g, 10 min). DNA was precipitated from the supernatant by adding absolute alcohol and centrifugation. The pellet of DNA was washed with 75% alcohol and finally dissolved in 100 µL of TE buffer and incubated at 37°C for 1 h. The quantity and quality of the DNA were appraised by electrophoresis on 1% Agarose-gels with a standard weight Lambda DNA (Sigma).

AFLP Protocol, Primer Screening and Marker Scoring

AFLPs analyses were performed using the restriction enzyme combination *Pst*I and *Mse*I according to the method of Vos *et al.* (1995). For each progeny, 100 ng of genomic DNA was digested in a 12.5 µL reaction with 2.5 units of *Pst*I and *Mse*I (Sigma) at 37°C for 6 h. The reaction contained 1.25 µL of 10 × NE buffer 4 and 0.15 µL of 100 × BSA. After digestion, 1.5 µL of 10 × T_4 DNA ligase buffer, 1.25 units of T4 DNA ligase and 1.25 units of *Pst*I and *Mse*I adapters were added to the reaction and the reaction was incubated at 37°C for another 3 h to ligate the adapters to the restricted DNA fragments.

After enzymatic restriction and adaptor ligation, an aliquot of 2.5 µL of the reaction was diluted 60 times with TE Buffer, and the diluted reaction was used for pre-amplification. The PCR reaction for the pre-amplification contained, 35 ng of *Pst*I and *Mse*I primers, 0.5 µL of dNTP mixture (10 mM), 0.5 µL of $MgCl_2$ stock solution (25 mM), 0.9 µL of 10 × PCR buffer (non-Mg^{2+}), 5 units of Taq DNA Polymerase in a total volume of 25 µL. The pre-amplification PCR was performed on PCR instrument (Eppendorf, Hamburg, Germany) with the cycling program of described by Vos *et al.* (1995).

All pre-amplification reactions were diluted 60 times with purified water and then 1 µL of this dilution was used in a 20 µL volume mixture for selective amplification. Each selective amplification reaction contained 5 ng of the *Pst*I primer, 30 ng of the *Mse*I primer, 0.4 µL of dNTP mixture (10 mM), 2 µL of the 10 × PCR buffer (non-Mg^{2+}), 1.6 µL of $MgCl_2$ solution (25 mM), and 0.4 units of Taq DNA Polymerase. Then the sample were amplified by PCR system and separated by 6% denatured polyacrylamide gels and DNA bands were visualized by silver staining. Primer combinations with three selective nucleotides (*Mse*I+3/*Pst*I+3) were screened to identify those that could amplify clear and unambiguous polymorphic fragments in both *Phalaenopsis* '462' and '20'.

AFLP marker fragments on the silver stained gels were manually scored for their absence (0) or presence (1). The distinguished clearly fragments were scored and recorded. There were a few ambiguous fragments in a few individuals. These fragments were resolved by assigning a blank score (-) for map construction. All AFLP markers were identified by primer combinations utilizing the primer notation (Vos *et al.*, 1995). The bands of polymorphic were named serially in the descending order of molecular weight. Recorded markers would be split into three groups according to the absence or presence within each parent. AFLP fragments that were present exclusively in the male parent were given the prefix M, while those present only in the female parent were given the prefix F, and those fragments appearing in both parents were given the prefix MF.

Data Analysis and Genetic Linkage Map Construction

In the pseudo-testcross configuration, Mendelian segregation was tested for all markers at a 5%

signification level by performing a chi square (χ^2) goodness-of-fit test to testcross (1:1) and intercross (3:1) marker ratios. The segregation of markers heterozygous in one parent was tested against a 1:1 ratio using a χ^2 test, while those heterozygous in two parents were tested against a ratio of 3:1. Those markers that did not segregate in the 1:1 or 3:1 ratio were treated as distorted ones.

AFLP markers were categorized into three types: (1) those manifesting segregation only in the male parent (*Phalaenopsis* '462'), (2) those manifesting segregation only in the female parent (*Phalaenopsis* '20'), and (3) those manifesting segregation in two parents. AFLP markers of the three types were used to construct the genetic linkage map for *Phalaenopsis*, and types 1, 3 for *Phalaenopsis* '462', types 2 and 3 for *Phalaenopsis* '20', following the methods of Grattapaglia and Sederoff (1994). Linkage maps were generated independently for each parent using JoinMap 3.0 (Van Ooijen and Voorrips, 2001) based on LOD scores (minimum LOD score of 3.0). The detection of linkage among AFLP markers were based on pair-wise recombination estimates with a threshold recombination fraction < 0.45 and a threshold LOD score > 3.0. Genetic distances were converted from recombination fractions into centiMorgans (cM) according to Kosambi (1943). Final linkage mapping was drawn using the software MapChart 2.1 (Voorrips, 2002).

Results

Polymorphism of AFLP Markers

Out of the 570 AFLP primer combinations tested, 71 showed polymorphisms that were highly reproducible and then used to evaluate the 88 F_1 progenies. Through the analysis of AFLP, the fragments size ranged from 50 to 1500 bp. These 71 primer combinations produced 865 amplification products, of which 416 showed polymorphic characteristics (Table 1). The number of polymorphic AFLP markers generated by each primer combination varied from 1 to 13, with an average of 5.86 polymorphic fragments per primer combination. Of these AFLP markers, 149 bands were amplified in the male parent and 226 in the female parent, and these markers were expected to segregate in a 1:1 ratio ($P < 0.05$). Forty-one markers were detected in two parents and they were predicted to segregate in a 3:1 ratio ($P< 0.05$) in the F_1 population.

Though the analysis of chi square, the 314 (75.48%) polymorphic markers showed a compatible fit to the anticipated segregation ratio of 1:1 or 3:1, and 102 (24.52%) polymorphic markers gave a significant segregation distortion from the anticipated ratio of 1:1 or 3:1 ($P = 0.05$). Out of 314 polymorphic markers, 175 (55.73%) were segregating in female, 109 (34.71%) through male and 30 (9.56%) through co-parental.

Map Construction

When the 416 AFLP markers were suffered to two-point linkage analysis using JoinMap 3.0, 292 markers were found suitable for linkage map construction. The remaining 124 markers (49% in female parent, 36% in male parent, and 15% in two parents) were not included because these markers did not accord with the linkage grouping or ordering thresholds (LOD score ≥ 3.0) of the pseudo-testcross mapping strategy in JoinMap 3.0. Two independent maps were constructed for each *Phalaenopsis* parental cultivar.

The genetic linkage map of *Phalaenopsis* '462' consisted of 122 markers (104 for the male parent, 18 for co-parents) in 15 linkage groups, and had a total genetic distance of 820.28 cM (Fig. 1). The length of the linkage groups varied from 3.67 cM to 168.96 cM, with an average of 6.72 cM between adjacent markers. The largest gap between two markers (37.8 cM) was found on Linkage Group 9. The average number of markers per linkage group was 8.13, ranging from 2 to 57 for each linkage group.

For *Phalaenopsis* '20', a total of 175 (165 for female parent, 10 for co-parents) markers were placed into 14 linkage groups, defining a total genetic distance of 878.29 cM (Fig. 2). The length of the linkage groups varied from 6.2 cM to 181.61 cM. The average distance between adjacent markers was 5.02 cM. The largest gap between two markers was found in Linkage Group 5. The number of markers for each linkage group varied from 2 to 107, with an average of 12.5 markers per linkage group.

Homologous Linkage Groups

AFLP loci that were heterozygous in both parents could help bridge homologous linkage groups of the co-parental map. In this study, 23 markers out of 41 shared by both parents were mapped on the parental maps, with 18 markers assigned on the male *Phalaenopsis* '462' map and 10 on the female *Phalaenopsis* '20' map. The homologous group nodes of two parental maps were formed in the five linkage groups. Markers MFP1-M17-450 and MFP13-M38-500 were detected in the linkage groups FLG-1 and MLG-1, markers MFP3-M19-600 in the linkage groups FLG-14 and MLG-1, and markers MFP11-M11-750 and MFP2-M10-500 in the linkage groups FLG-9 and MLG-3 (Fig. 3). Therefore, homologous relationships could exist in the linkage groups of bi-parentals.

Discussion

Compared with other markers, AFLP is a dominant PCR-based marker, requiring relatively little template DNA and little prior knowledge of the target template DNA sequence, and reveals high levels of polymorphism in many species.

Table 1: The number of polymorphic fragments and mapped fragments generated by each of 71 *Pst*I+3/*Mse*I+3 primer combinations in the development of the genetic linkage maps of *Phalaenopsis* '462' × *Phalaenopsis* '20'

Primer combinations	Primer code	Number of polymorphic fragments	Number of mapped fragments	Primer combinations	Number of polymorphic fragments	Number of mapped fragments	Mapped markers
P-GAC+M-CAG	P1-M3	6	5	P-GCT+M-ACT	P5-M11	8	6
P-GAC+M-CTA	P1-M6	6	4	P-GCT+M-ACA	P5-M27	3	2
P-GAC+M-AAC	P1-M8	5	3	P-GCT+M-GAC	P5-M32	7	5
P-GAC+M-AAG	P1-M9	4	2	P-GCT+M-GCG	P5-M38	13	9
P-GAC+M-ACC	P1-M10	5	4	P-GCG+M-CAG	P6-M3	4	4
P-GAC+M-ACT	P1-M11	5	3	P-GCG+M-AAC	P6-M8	5	2
P-GAC+M-ATC	P1-M16	5	4	P-GCG+M-AAG	P6-M9	6	3
P-GAC+M-ATG	P1-M17	7	4	P-GCG+M-ACT	P6-M11	9	6
P-GAC+M-CCG	P1-M18	4	1	P-GCG+M-AGC	P6-M12	9	6
P-GAC+M-CCT	P1-M19	4	4	P-GCG+M-ATC	P6-M16	7	6
P-GAC+M-CTG	P1-M21	3	3	P-GCG+M-CTC	P6-M20	1	1
P-GAC+M-CGA	P1-M22	8	7	P-GCG+M-GAG	P6-M33	4	4
P-GAC+M-ACA	P1-M27	6	4	P-GCG+M-GTA	P6-M36	4	2
P-GAC+M-ATT	P1-M30	4	3	P-GCG+M-GTT	P6-M37	6	5
P-GAG+M-CAG	P2-M3	7	6	P-GGA+M-ACC	P10-M10	4	4
P-GAG+M-AAG	P2-M9	4	2	P-GGA+M-ACT	P10-M11	9	9
P-GAG+M-ACC	P2-M10	8	5	P-GGC+M-AAC	P11-M8	12	7
P-GAG+M-ACT	P2-M11	4	4	P-GGC+M-AAG	P11-M9	13	11
P-GAG+M-AGT	P2-M14	8	4	P-GGC+M-ACC	P11-M10	3	3
P-GAG+M-ATC	P2-M16	1	1	P-GGC+M-ACT	P11-M11	12	10
P-GAG+M-ATG	P2-M17	7	5	P-GGC+M-AGG	P11-M13	10	7
P-GAG+M-CCG	P2-M18	6	5	P-GGC+M-ATC	P11-M16	2	2
P-GAG+M-CTG	P2-M21	12	4	P-GGC+M-GAC	P11-M32	9	6
P-GAG+M-CGA	P2-M22	11	6	P-GGC+M-GAG	P11-M33	6	6
P-GAG+M-CGG	P2-M24	9	6	P-GGC+M-GCG	P11-M38	2	1
P-GAG+M-GAG	P2-M33	7	3	P-GGT+M-AAG	P12-M9	2	2
P-GAG+M-GAT	P2-M34	6	4	P-GGT+M-ACA	P12-M27	5	4
P-GAG+M-GTA	P2-M36	3	2	P-GGT+M-ACG	P12-M28	4	3
P-GAG+M-GCG	P2-M38	11	8	P-GGT+M-GAC	P12-M32	5	4
P-GAT+M-CAG	P3-M3	5	3	P-GGT+M-GTT	P12-M37	10	9
P-GAT+M-CCA	P3-M5	4	2	P-GGT+M-GCG	P12-M38	5	5
P-GAT+M-CCG	P3-M18	1	0	P-GAA+M-CAG	P13-M3	4	3
P-GAT+M-CCT	P3-M19	3	2	P-GAA+M-CAT	P13-M4	3	2
P-GAT+M-CTC	P3-M20	6	0	P-GAA+M-CTG	P13-M21	2	1
P-GAT+M-CTG	P3-M21	5	2	P-GAA+M-GCG	P13-M38	6	5
P-GCT+M-ACC	P5-M10	4	2	-	-	-	-

This molecular marker system has been used in the studies of genetic diversity and gametophyte selection of *Phalaenopsis* (Hsu *et al.,* 2008; Chang *et al.,* 2009, 2010; Gawenda *et al.,* 2012). The AFLP technique has been widely used for linkage mapping in many plants including sweetpotato (Kriegner *et al.*, 2003), velvetbean (Capo-Chichi *et al.*, 2004), apple (Kenis and Keulemans, 2005), wintersweet (Chen and Chen, 2010), crape myrtle (He *et al.*, 2014), and *Eucommia ulmoides* (Wang *et al.,* 2014). A high level of DNA polymorphism detected by AFLP technique in *Phalaenopsis* allowed us to construct its genetic linkage map using F_1 progenies.

AFLP markers were sensitive to segregation distortion and highly distorted markers had been discovered in many species (Behrend *et al.*, 2013; Chen *et al.*, 2013; Han *et al.*, 2002), which was supposed to be one of the forces for biological evolution (Konishi *et al.*, 1990). The segregation distortion was often much higher in inter-specific populations than in intra-specific populations (Myburg *et al.*, 2003) and caused from biological reasons such as gametes selection, faulty chromosome pairing and so on (Zhang *et al.*, 2010). There were an average 24.42% of the distorted segregation in our study of *Phalaenopsis*, which was similar to 22.61% in crape myrtle (He *et al.*, 2014), 24.8% in lily (Abe *et al.*, 2002) and 23.5% in rhododendron (Dunemann *et al*., 1999), and less than 34.8% in wintersweet (Chen and Chen, 2010). Highly distorted segregation markers used to construct genetic maps did not affected marker order and map length (Hackett and Broadfoot, 2003), so some genetic maps were constructed without removing the segregation markers (Ky *et al.*, 2000; Han *et al.*, 2002; Yan *et al.*, 2005; Behrend *et al.*, 2013). Here, maybe the biological cause was the main reason. Most of *Phalaenopsis* are heterozygous at the gene, including both the parents. Thus, further researches with intraspecific crosses, larger progenies and more molecular markers would be helpful to study the segregation distortion in *Phalaenopsis*. Considering the addition of distorted markers could increase the identification of regions of interest in further study of *Phalaenopsis*,

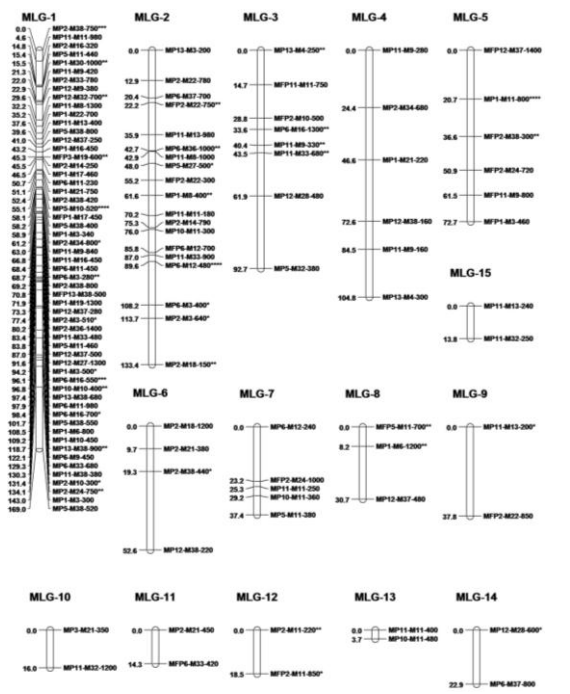

Fig. 1: Genetic linkage map of *Phalaenopsis* '462' based on the segregation of AFLP markers in a population of 88 F_1 progenies. For each linkage group, the names of AFLP markers are shown on the right side and the marker intervals in Kosambi centimorgans (cM) and their map position shown on the left side. For details of AFLP marker nomenclature see Table 1. Asterisks indicate distorted segregation of markers (χ^2 test) *p = 0.05, **p = 0.01, ***p = 0.005, ****p = 0.001, *****p = 0.0005

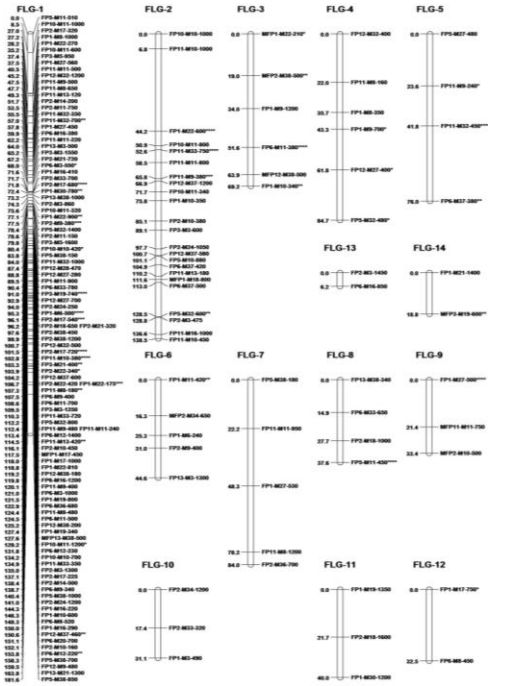

Fig. 2: Genetic linkage map of *Phalaenopsis* '20' based on the segregation of AFLP markers in a population of 88 F_1 progenies. For each linkage group, the names of markers are shown on the right side and the marker intervals shown in Kosambi centimorgans (cM) their map position (cM) on the left side. For details of AFLP marker nomenclature see Table 1. Asterisks indicate distorted segregation of markers (χ^2 test) *p = 0.05, **p = 0.01, ***p = 0.005, ****p = 0.001, *****p = 0.0005

the distorted markers were not discarded and marked with asterisks on the construction of genetic map in this study.

Nineteen link groups were anticipated to be mapped corresponding to the haploid chromosomes number for genus *Phalaenopsis* (2n = 38). However, only 15 linkage groups were obtained in the male map (Fig. 1) and 14 in the female map (Fig. 2) in this study. Similar situation had been reported in other plant species (Mignouna *et al.*, 2002; Chen and Chen, 2010; Xue *et al.*, 2010; Zheng *et al.*, 2013). In this paper, a low number of markers and small population were related to small linkage groups and unlinked markers. With additional markers and larger population, a much more comprehensive genetic map of *Phalaenopsis* would be constructed.

The wide marker space of 20 or even 50 cM may be optimal for QTL mapping scanning (Darvasi *et al.*, 1993). In this study, AFLP markers with the average recombination distance were 6.72 cM and 5.02 cM, respectively for parental linkage maps that provided a favorable situation for QTL scanning.

The clustering of many markers loci to one or two linkage groups intensively seems a common phenomenon in AFLP genetic linkage maps (Ouédraogo *et al.*, 2002; Strommer *et al.*, 2002). This occurred in FLG-1 and MLG-1 of *Phalaenopsis* map. One of the reasons for this intensive clustering phenomenon may be the higher sensitivity of the AFLP technique because the degraded recombination rate near centromere or telomere, non-equal of restriction enzyme sites and high repeat factor could cause these intensive clustering markers (Strommer *et al.*, 2002). AFLP markers from *Eco*RI/*Mse*I restriction enzymes were found to concentrate often in the vicinity of the centromere of chromosome due to centromeric suppression of recombination (Haanstra *et al.*, 1999; Bonnema *et al.*, 2002), while contrarily the polymorphic markers from PstI/MseI restriction enzymes were distributed more equally than that marker from *Eco*RI/*Mse*I restriction enzymes in the genome (Pradhan *et al*., 2003). However, *Pst*I/*Mse*I were used as restriction enzymes in this study. High density clustering of markers still appeared in the linkage groups in FLG-1 and MLG-1. It has been argued that high density clustering may be the results of the recombination or too small mapping population (De la Rosa *et al.*, 2003).

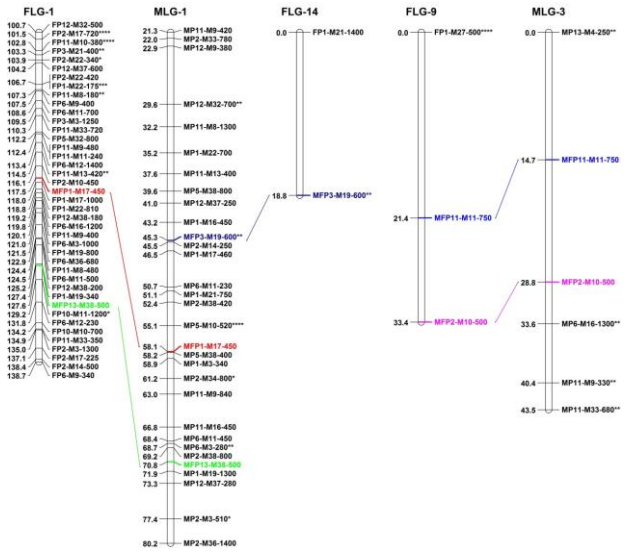

Fig. 3: Homologous linkage groups of both maps are presented side by side. For each linkage group, the names of markers are shown at the left and the marker intervals shown in Kosambi centimorgans (cM) their map position (cM) at the right. For details of AFLP marker nomenclature see Table 1. Asterisks indicate distorted segregation of markers (χ^2 test) *p = 0.05, **p = 0.01, ***p = 0.005, ****p = 0.001, *****p = 0.0005

Conclusion

A preliminary genetic linkage map was constructed for *Phalaenopsis* by AFLP markers, a species that was relatively lacking the genetic information. To our knowledge, this was the first linkage map for *Phalaenopsis*, which may serve as a tool in QTL analysis, molecular marker assisted selection, and map-based cloning in further study, especially the linkage between molecular markers and valuable genes was the premise for cloning gene such as fragrance, growth habit and flower color, or other single gene traits on the maps. However, this map was not saturated, more additional markers and larger population needed to be used to construct a much more comprehensive genetic map of *Phalaenopsis*.

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