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Physical mapping of 45S and 5S rDNA and telomeric repeat loci in eight diploid hyacinth cultivars

YUAN-HAO HE[1,2], XIAO-QIAN SU[1,3], ZI-HAN XU[1,4] & FENG-RONG HU[1,5*]
[1] *College of Landscape Architecture, Nanjing Forestry University, Nanjing 210037, China*
[2] *871375753@qq.com; https://orcid.org/0000-0002-5087-9987*
[3] *871375753@qq.com; https://orcid.org/0000-0003-0763-3831*
[4] *406922521@qq.com; https://orcid.org/0000-0003-2721-7683*
[5] *hufengrong2003@sina.com; https://orcid.org/0000-0003-2537-1150*
[*] *Author for correspondence*

Abstract

Hyacinth, one of the five best-selling bulbous flowers in the world, is a favored flower of garden landscapes in the spring. Hyacinth has a single origin, but there are many horticultural varieties. Physical mapping of ribosomal DNA (rDNA) and telomeric repeat loci by fluorescence in situ hybridization (FISH) is helpful to understand the genetic relationship and chromosome inheritance in hyacinth breeding. In this study, 45S rDNA, 5S rDNA and telomeric repeats were used as probes, the mitotic metaphase chromosomes of 8 diploid hyacinth cultivars were analyzed by FISH location, and their karyotype parameters were analyzed by Q-type cluster analysis. The results showed that two 45S rDNA loci were detected in all eight hyacinth cultivars, four 5S rDNA loci were detected in 'Gypsy Queen', and three were detected in other cultivars. In addition to the presence of telomere loci at both ends of chromosomes, seven telomere loci were detected in 'Gypsy Princess' and 'Yellow Stone', and eight telomere loci were detected in other cultivars. At the same time, when the euclidean distance between groups was 20, the eight hyacinth cultivars were clustered into three groups ('Yellow Stone', 'Red Pearl' and 'Pink Pearl'), and 'Gypsy Prince' was a single group; the other four cultivars were in one group. The distribution of 45S rDNA loci in diploid hyacinth cultivars was relatively conservative, while the locus number and distribution of 5S rDNA and telomeric repeats were highly polymorphic. The FISH physical mapping in this study provides a basic understanding of the karyotype evolution of hyacinth species, which could increase the understanding of the evolutionary history and phylogenetic relationships of hyacinths.

Keywords: 45S rDNA, 5S rDNA, Telomeric repeats, Fluorescence in situ hybridization, Karyotype parameters, Genetic relationship, Hyacinth

Introduction

Hyacinth (*Hyacinthus orientalis* Linnaeus (1753: 317) is a well-known bulbous ornamental plant species belonging to the genus *Hyacinthus* Linnaeus (1753: 317) and the family Hyacinthaceae. Hyacinth originates from the eastern coast of the Mediterranean and Asia Minor, and its flowers are rich in color, beautiful in shape and fragrant in smell. In addition, hyacinth is cultivated in various ways. It can be used to decorate flower borders and can also be hydroponically cultivated or grown as cut flowers for viewing (Hu *et al.* 2011, Xie *et al.* 2017). Modern horticultural hyacinth has a simple origin. Although there are one species and three cultivars, only *H. orientalis* is a prevailing horticultural flower, and all commercial species are derived from this species through gene mutation or intraspecific hybridization (Hu *et al.* 2012). In view of the close genetic relationship and the fuzzy degree of evolution among cultivars, carrying out unified and scientific cultivar classification is a bottleneck that increases the difficulty of specific cultivar breeding.

Chromosome characteristics and variation in different taxa can be analyzed by cytogenetic research to infer the degree of species evolution and interspecific genetic relationships, and chromosome identification is its basis (Ilnicki 2014, Liu *et al.* 2018). In recent years, research on hyacinth has mostly focused on several aspects, such as biological properties, cultivation, breeding, and secondary metabolism (Miyamoto *et al.* 2015, Hu *et al.* 2015, Alexandre *et al.* 2017, Xie *et al.* 2018). Research on hyacinth chromosomes can be traced back to the 1970s. Chromosome karyotype, banding and in situ hybridization techniques were developed in the 1960s-1970s and are widely used in plant research;

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however, there are few cytological research reports on *H. orientalis*. Van Tuyl (1982) reported the chromosome numbers of 14 cultivars and 4 wild accessions of *H. orientalis*. Hu *et al.* (2011) determined the karyotypes of five diploid (2x=16) cultivars with diverse flower colors using C-banding and 45S rDNA fluorescence in situ hybridization (FISH) analysis. In general, research on hyacinth cytogenetics is still in the initial stages.

Traditional karyotype analysis is mainly based on the length and arm ratio of chromosomes, so it is difficult to identify each chromosome of the research object. FISH of chromosome-specific sequences provides an effective method for the analysis of karyotype and genome changes (Chester *et al.* 2010, Shibata *et al.* 2016). FISH technology directly locates the repeat sequences of rDNA loci on chromosomes by fluorescent labeling to provide effective cytological markers for chromosomes carrying rDNA loci (Xu *et al.* 2010, Wang *et al.* 2016). rDNA is a highly conserved tandem repeat in the biological genome. In the study of cytogenetics, the number and distribution of rDNA loci provide effective identification markers for karyotype analysis (Thomas *et al.* 2001), FISH technology with rDNA as probes has been applied to chromosome recognition in many plants (Yukio *et al.* 2010). Hont *et al.*(2000) used rDNA fish and Gish technology to accurately distinguish the chromosomes of four cultivated banana genomes. Yukio *et al.*(2010) used 5S rDNA and 25 rDNA as probes to perform fluorescence in situ hybridization on 6 species of *Brachiaria eruciformis*, and identified homologous chromosomes between species. The telomere DNA sequence of eukaryotes was first isolated from Arabidopsis thaliana in 1988 (Richards 1988). In some plants, telomere repeat site can be located not only at the ends of chromosomes, but also inside chromosomes, which is called interstitial telomere repeat (ITR). Fuchs (1995) used telomere repeats as probes to locate the chromosomes of *Pinus sylvestris*, founding that in addition to the ends of chromosomes, some chromosome centromeres also had site distribution. *Morus notabilis* also had the phenomenon that telomere sites were located at the centromere of chromosomes (Li 2015).

Tandem repeats mainly include ribosomal DNA (rDNA), centromere sequence and telomere sequence, which play an important role in maintaining the spatial structure of chromosomes and regulating gene expression. Fluorescence in situ hybridization (FISH) technology, using tandem repeats as probes to locate FISH sites on chromosomes, can provide accurate and effective cytological markers for karyotype analysis, especially for plants with small and similar morphology chromosomes, which is an important means of karyotype analysis.

At present, FISH physical mapping has been applied to bulbous flowers such as lilies (Cao *et al.* 2019) and tulips (Lan *et al.* 2018) for karyotype analysis and rDNA localization. However, to our knowledge, the research on the physical location of hyacinth is scarce. At present, there are diverse hyacinth horticultural varieties in the market, and it is extremely difficult to distinguish except for color. For varieties with different ploidy, they can be distinguished by karyotype analysis preliminarily, while for varieties with the same color and ploidy, it is essential to use multi probe FISH technology for physical mapping. In this study, the mitotic metaphase chromosomes of 8 commercial hyacinth (2x) varieties were located by physical mapping with 45S rDNA, 5S rDNA and Arabidopsis-type telomeric repeat probes, aiming to (i) construct FISH karyotype maps of 8 diploid varieties and analyze the chromosome composition and inheritance of each variety and (ii) explore the genetic relationship and degree of differentiation among varieties by cluster analysis.

Materials and methods

Plant materials and probes:—Eight diploid hyacinth cultivars of five colors were used for physical mapping. The bulbs used in the study were imported from the CBTC International Trade b.v. of Holland. These cultivars included ‘Gypsy Queen’, ‘Purple Sensation’, ‘Pink Pearl’, ‘Gypsy Princess’, ‘Blue Pearl’, ‘Odysseus’, ‘Yellow Stone’, and ‘Red Pearl’ (Figure 1).

The probes used in the research were from Nanjing Boqiao Biotechnology Co., Ltd. (Nanjing, Jiangsu Province). 45S rDNA was labeled with digoxigenin-11-dUTP as the probe, 5S rDNA was labeled with biotin-16-dUTP as the probe, and the telomeric DNA probe was labeled with digoxigenin or biotin.

Chromosome preparation and parameter measurements:—Methods for chromosome-spread preparation followed those of Hu *et al.* (2012). Ten water-cultivated root tips of each hyacinth cultivar were randomly selected and dissociated with acetic acid (45%) for 2 h, and an alkaline solution was applied to compress the red-stained tablet. Then, the samples were observed under an Olympus 60* objective lens microscope, and images were captured with a DP72 system (Olympus, Japan).

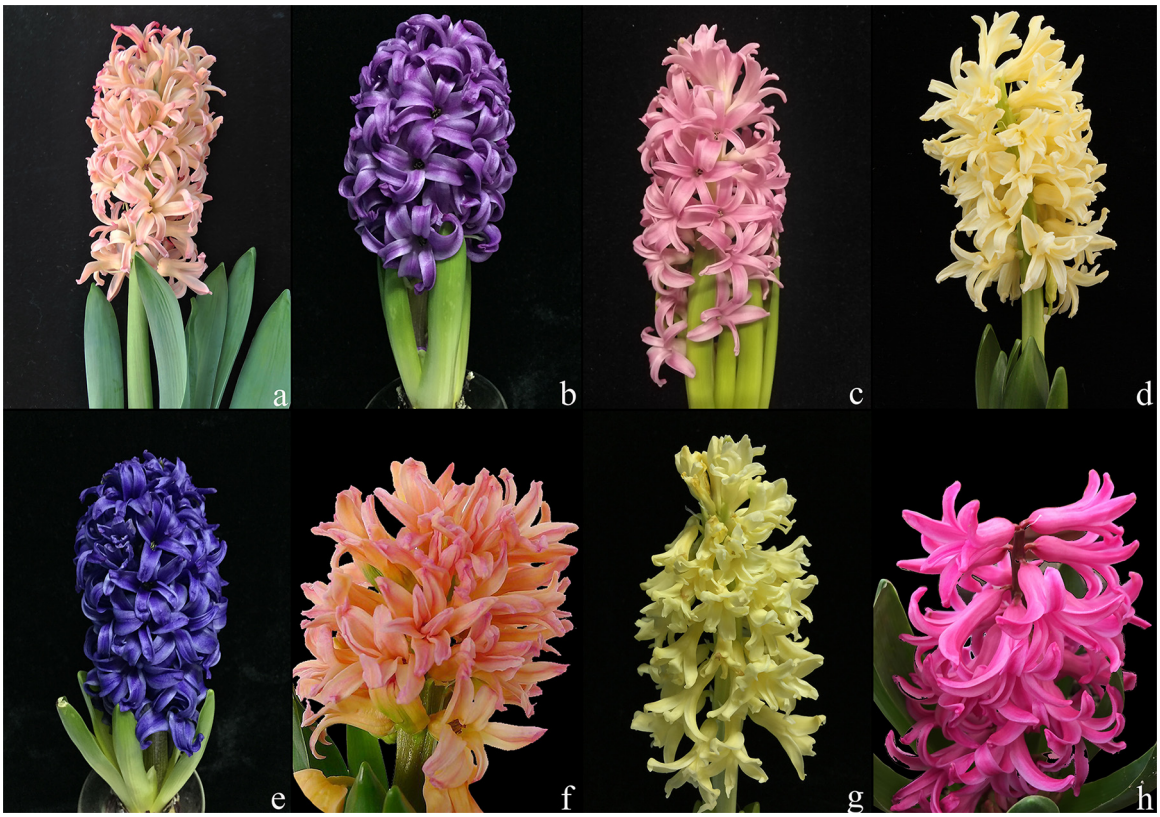

FIGURE 1. Eight diploid hyacinth cultivars

a.'Gypsy Queen'; b.'Purple Sensation' c.'Pink Pearl' d.'Gypsy Princess' e.'Blue Pearl' f.'Odysseus' g.'Yellow Stone' h.'Red Pearl'

Five premium-quality karyotype photos were selected, Photoshop software (Adobe, USA) was used to cut and match chromosomes from the same source, and the long and short arms of chromosomes were measured by Auto CAD2007 software (Autodesk, USA). The chromosome karyotype parameters were calculated by Excel software.

The chromosome type, karyotype analysis and calculation method used for related parameters, as well as the asymmetry coefficient of karyotype (AsK%=total length of long arm of chromosome / total chromosome length), can be found in previous studies (Arano 1963, Levan *et al.* 1964, Stebbins *et al.* 1971, Li *et al.* 1985).

Fluorescence in situ hybridization:—Chromosome preparation. The methods of root tip pretreatment, fixation, dissociation and compression are the same as those of karyotype parameter measurement. Ten root tips of hyacinth cultivars were randomly selected and dissociated with acetic acid (45%) for 2 h. Acetic acid (45%) was used to compress the slides, and then, the slides were uncovered with liquid nitrogen after microscopic examination, dehydrated with absolute ethanol, and air dried until use.

The enzymolysis method was performed as described in previous studies (Lim, 2000; Lim *et al.*, 2002) with minor modifications. The slides were pretreated with RNase I solution (100 μg/μL) for 1 h and pepsin solution (4%) for 10 min, both at 37 ℃, followed by formaldehyde (4%) for 10 min at room temperature, gradient dehydration in 70%, 95% and absolute ethanol for 3 min each and air dried until standby.

The FISH procedure and washing and detection of the probe were carried out by the methods described by Fang *et al.* (2014) with minor modifications. The hybridization mixture of each slide was 20 μL: 10 μL of 10% DFA, 2 μL of 20× SSC, 4 μL of 50% DS, 2 μL of 45S rDNA (Arabidopsis-type telomeric DNA, 2 μL), and 2 μL of 5S rDNA (ddH_2O, 2 μL). After overnight hybridization at 37°C in a humid box, slides were washed at room temperature in 2x SSC 2~3 times for 5 minutes each time and then washed with distilled water for 5 min. Slides were washed with 1×PBS three times and counterstained with 20 μL of Vectashield containing DAPI (4′,6-diamidino-2-phenylindole). Hybridization loci were observed under a fluorescence microscope (BX51, Olympus, Japan), and the images were captured with a DP72 system (Olympus, Japan).

Cluster analysis:—The cytological characteristics of 8 hyacinth cultivars were studied by between-group linkage with SPSS software. Q-type cluster analysis was conducted using ten indexes: average arm ratio (AR), the longest chromosome length/the shortest chromosome length (LC/SC), the proportion of chromosomes with an arm ratio value>2 (AR>2), asymmetrical karyotype coefficient (ASK), sum of the centromere index (CI), chromosome relative

length range (LC-SC), sum of the reciprocal of the arm ratio (1/AR), average of the reciprocal of the arm ratio, metacentric chromosome number (m), sum of the submetacentric chromosome (sm) and subtelocentric chromosome (st) numbers, and karyotype type (2B is quantized as 0, and 2C is quantized as 1).

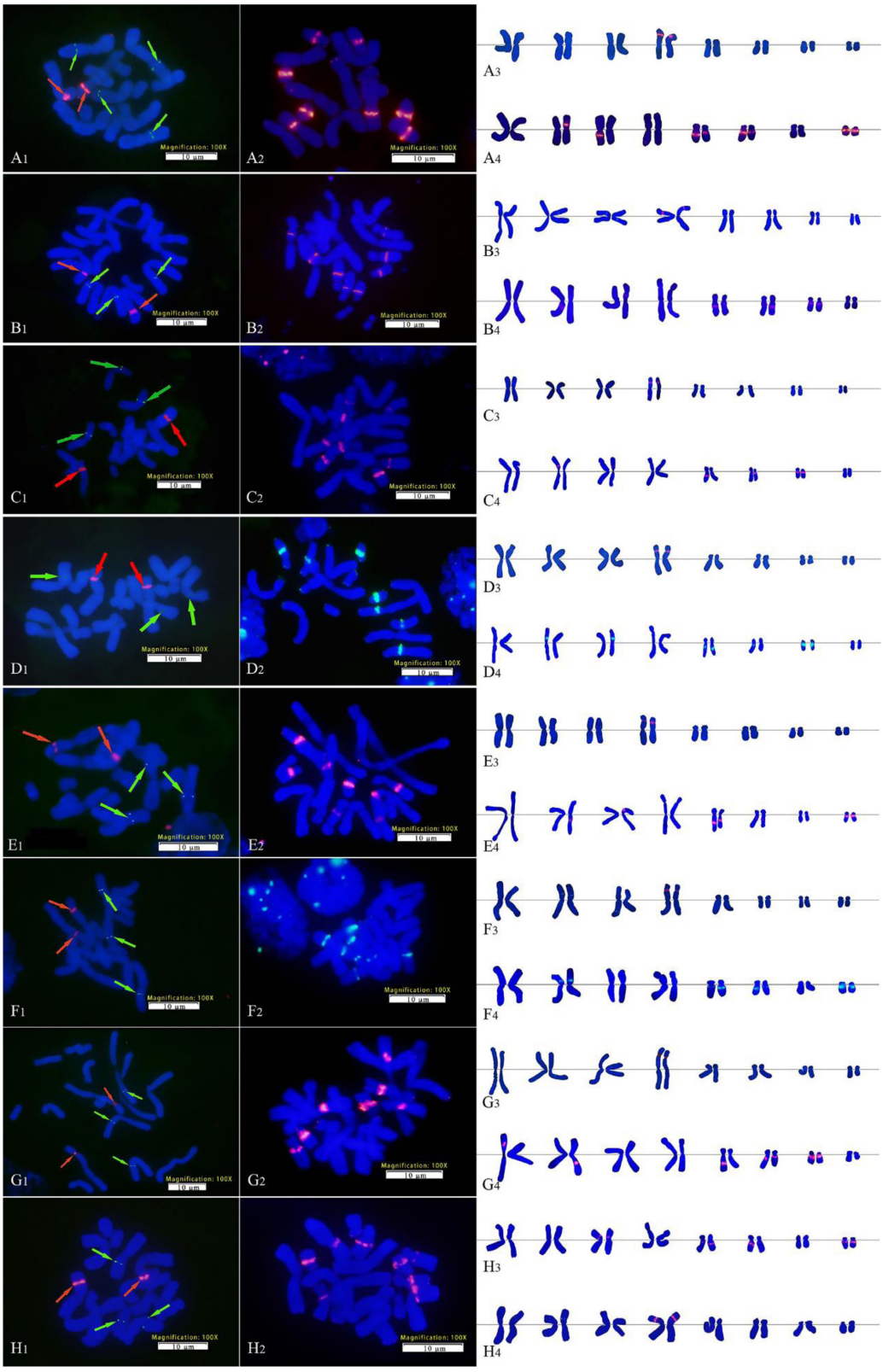

FIGURE 2. Results of FISH physical mapping on metaphase chromosomes of hyacinth

1. The distribution of 45S rDNA (red arrow) and 5S rDNA (green arrow) signals on the chromosomes; 2. The distribution of telomeric repeats signals on the chromosomes; 3. Chromosome karyotype with 45S rDNA and 5S rDNA; 4. Chromosome karyotype with telomeric repeats.

A. 'Gypsy Queen' B.'Purple sensation' C.'Pink pearl' D.'Gypsy princess' E.'Blue pearl' F.'Odysseus' G.'Yellow stone' H.'Red pearl'

Results

FISH location of 45S and 5S rDNA loci:—The results of FISH with 45S and 5S rDNA probes (Figure 2, 3) showed that a pair of 45S rDNA locus was detected in the 8 diploid hyacinth cultivars and were located at the secondary constriction of the satellite (SAT) chromosome. Four 5S rDNA loci were detected in 'Gypsy Queen' on the long arm of chromosomes 2 and 5 and the short arm of chromosomes 4 and 6. In the other seven cultivars, three 5S rDNA loci were detected, of which two 5S rDNA loci were located in the short arms and one was detected in the long arm in five of the seven cultivars. In 'Purple Sensation', 5S rDNA loci were observed on the short arms of chromosomes 4 and 5 and the long arm of chromosome 6. In 'Pink Pearl', 5S rDNA loci were detected on the short arms of chromosomes 3 and 5 and the long arm of chromosome 6; 'Blue Pearl' possessed loci on the long arm of chromosome 2 and the short arms of chromosomes 4 and 6. In 'Odysseus', loci were observed on the short arms of chromosomes 3 and 4 and the long arm of chromosome 5. In 'Red Pearl', 5S rDNA loci were observed on the short arms of chromosomes 2 and 4 and the long arm of chromosome 5. In the remaining two cultivars, two 5S rDNA loci were located in the long arms, and one was located in the short arm: loci were detected on the long arms of chromosomes 2 and 6 and the short arm of chromosome 5 in 'Gypsy Princess' and on the long arms of chromosomes 1 and 3 and the short arm of chromosome 6 in 'Yellow Stone'.

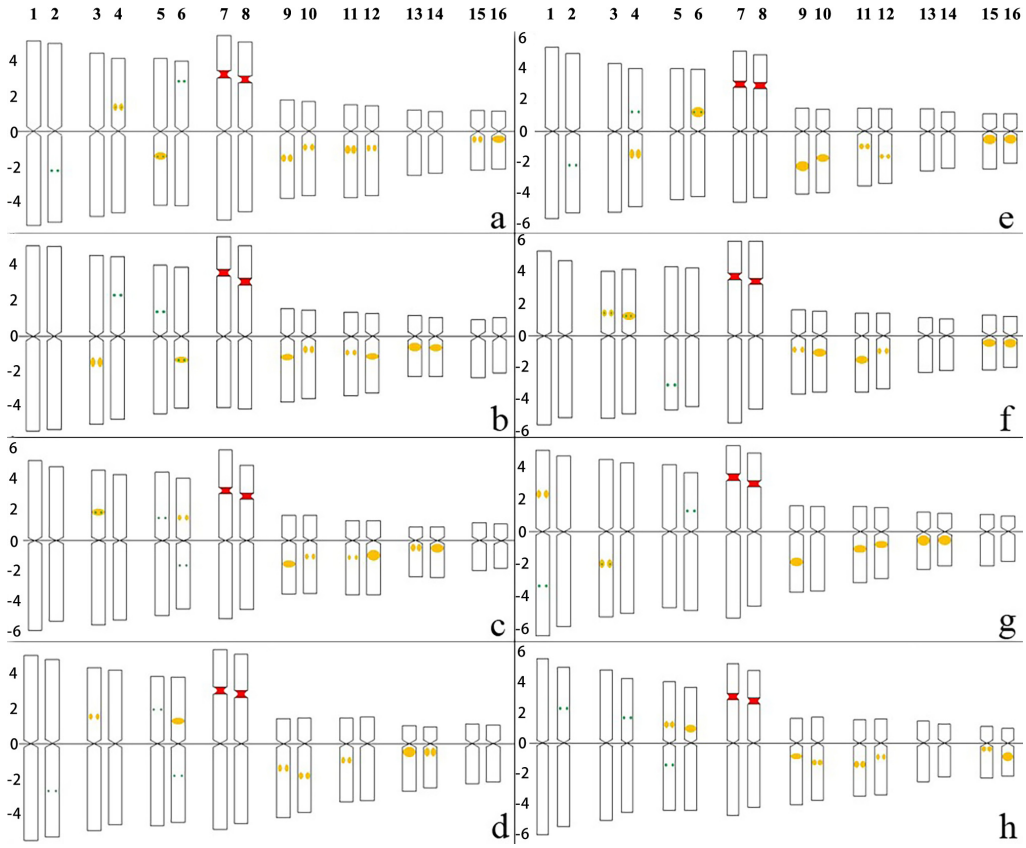

FIGURE 3. FISH karyotype pattern diagram of 8 diploid hyacinth cultivars

a.'Gypsy Queen'; b.'Purple sensation' c.'Pink pearl' d.'Gypsy princess' e.'Blue pearl' f.'Odysseus' g.'Yellowstone' h.'Red pearl'

Red point: 45S rDNA loci; Green point: 45S rDNA loci; Yellow point: ITR sites

FISH location of telomeric repeats:—Fluorescence in situ hybridization using Arabidopsis-type telomeric repeat 5'-(TTTAGGG)n-3' as a probe (Figure 2, 3) showed that interstitial telomere repeats site (ITR) in *H. orientalis* not only existed at both ends of each chromosome but also had loci on the arms of chromosomes, and their loci intensities were strong. In this study, ITR sites at both ends of chromosomes were omitted, and only the ITR sites on the long and short arms of chromosomes were analyzed. In 'Gypsy Princess' and 'Yellow Stone', seven ITR sites were detected on the long and short arms of chromosomes, and in other cultivars, eight ITR sites were detected. Among them, only 'Yellow Stone' had a ITR site on the short arm of the first pair of chromosomes, and no ITR site was detected on the first pair of the chromosome arms of other cultivars; at least one chromosome of the second pair in other cultivars except 'Red Pearl' contained a ITR site.

No ITR site was detected on the arms of chromosomes 15 and 16 in 'Purple Sensation', 'Pink Pearl', 'Gypsy Princess' and 'Yellow Stone'. Similarly, there was no ITR site on the arms of chromosomes 13 or 14 in 'Gypsy Queen', 'Blue Pearl', 'Odysseus', and 'Red Pearl'. We speculated that there are telomere-related genes in the chromosome arm of *H. orientalis*.

Comparative karyotyping of eight hyacinth cultivars by FISH:—According to the existing karyotype data and FISH results, chromosome FISH maps of 8 hyacinth cultivars were drawn (Figure 3, Table 2). From the karyotype pattern diagram, we found no other probe hybridization loci on the SAT chromosome except for the 45S rDNA . In the figure 3 is observed the co-localization of the loci of the 5S rDNA with the ITR sites in 6 of the 8 hyacinth cultivars. There were two coincidence loci in 'Gypsy Queen' and 'Odysseus'. One coincidence locus was detected in 'Purple Sensation', 'Pink Pearl', 'Blue Pearl', and 'Yellow Stone'. The genetics of 'Gypsy Queen' and 'Odysseus' were similar.

Karyotype parameters and cluster analysis:—The karyotype parameters of 8 diploid hyacinth cultivars published by our research group (Su *et al.*, 2019; Su *et al.*, 2020; He *et al.*, 2021) were calculated and listed (Table 1) for cluster analysis with SPSS software.

TABLE 1. Karyotype parameters of 8 hyacinth cultivars.

Cultivars	Average AR	LC/SC	Chromosome number (AR>2)	ASK (%)	Sum of CI	LC-SC	Sum of 1/AR	m type chromosome number	sm and st type chromosome number	Karyotype type
'Gypsy Queen'	1.79	3.49	8	59.24	6.04	7.23	10.24	6	10	2B
'Purple Sensation'	1.85	3.67	8	58.92	6.06	7.60	10.23	8	8	2B
'Pink Pearl'	1.94	4.36	6	59.85	5.84	8.36	9.66	6	10	2C
'Gypsy Princess'	2.03	3.56	8	61.31	5.67	7.39	9.27	6	10	2B
'BluePearl'	1.86	3.78	8	59.76	6.06	7.86	9.96	8	8	2B
'Odysseus'	1.78	3.76	6	59.18	6.06	7.64	10.17	8	8	2B
'Yellow Stone'	1.79	4.61	8	60.63	5.90	8.66	9.63	6	10	2C
'Red Pearl'	1.84	4.07	6	59.49	5.99	8.39	9.93	6	10	2C

According to the cytological indexes of H. *orientalis*, 8 cultivars were analyzed by Q-type cluster analysis with SPSS 21.0 software (Figure 4). At a euclidean distance of 20, the eight cultivars were clustered into three groups. There were 4 cultivars in Group I: 'Purple Sensation', 'Blue Pearl', 'Odysseus' and 'Gypsy Queen'. 'Yellow Stone', 'Red Pearl' and 'Pink Pearl' comprised Group II. 'Gypsy Princess' alone was separated into Group III, of which Group II was relatively close to Group I in terms of genetic relatedness.

TABLE 2. The location of the rDNA loci and ITR sites in eight hyacinth species.

Cultivars	45S rDNA loci	5S rDNA loci	ITR sites
'Gypsy Queen'	7,8	2,4,5,6	4,5,9,10,11,12,15,16
'Purple Sensation'	7,8	4,5,6	3,6,9,10,11,12,13,14
'Pink Pearl'	7,8	3,5,6	3,6, 9,10,11,12,13,14
'Gypsy Princess'	7,8	2,5,6	3,6, 9,10,11,13,14
'Blue Pearl'	7,8	2,4,6	4,6,9,10,11,12,15,16
'Odysseus'	7,8	2,4,6	3,4,9,10,11,12,15,16
'Yellow Stone'	7,8	1,3,6	1,3,9,11,12,13,14
'Red Pearl'	7,8	2,4,5	5,6, 9,10,11,12,15,16

Five cultivars exhibited isomorphic location phenomena. On 'Pink Pearl' chromosome 6, 'Gypsy Princess' chromosome 6, 'Yellow Stone' chromosome 1 and 'Red Pearl' chromosome 5, 5S rDNA loci were located in the long arm, and ITR site were located in the short arm. On 'Blue Pearl' chromosome 4, 5S rDNA loci were located in the short arm, and ITR site were located in the long arm. 'Pink Pearl', 'Gypsy Princess', 'Yellow Stone' and 'Red Pearl' were closely related.

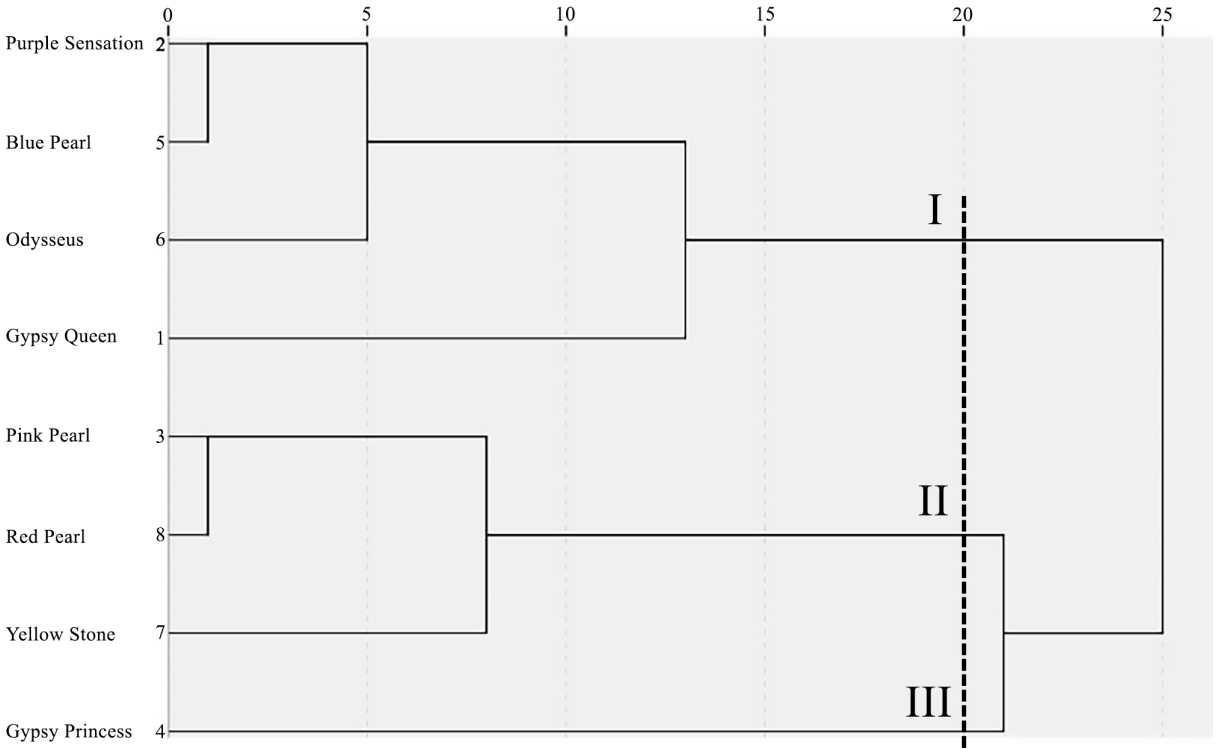

FIGURE 4. Dendrogram of eight hyacinths species using between-groups linkge
At the Euclidean distance of 20, the eight were clustered into group I, II and III.

Discussion

FISH mapping of 8 hyacinth cultivars:—45S rDNA loci usually refer to NORs, which are related to nucleolus formation, generally at the secondary constriction of chromosomes (Lan *et al.* 2016). However, NORs do not exist only at the secondary constriction (Li *et al.* 1996); in other words, not all plant 45S rDNA loci exist only on SAT chromosomes. In mysorethorn (*Caesalpinia decapetala*), 45S rDNA loci were also detected at the short arm ends of several chromosomes except the secondary constriction of the SAT chromosomes (She *et al.* 2019). The 45S rDNA locus of most *Rosa* plants is located at the end of the short arm of chromosomes (Tan *et al.* 2019). Some studies have suggested that 45S rDNA is easier to change than 5S rDNA during plant evolution (Liu *et al.* 2003, Xu *et al.* 2010). In this study, a pair of 45S rDNA locus was detected in 8 diploid hyacinth cultivars located at the secondary constriction of the SAT chromosomes. The loci were relatively stable, but the intensities were different. We speculated that the size heteromorphism of 45S rDNA loci was different in those varieties, or the two SAT chromosomes stemmed from different hyacinth provenances.

As the localization of the 45S rDNA probe is conserved in the investigated cultivar, both the 5S rDNA probe and telomere probe revealed more variable loci. Four 5S rDNA loci, located on chromosomes 1~6, were detected in 'Gypsy Queen', and 3 were detected in the other cultivars, with the intensities being relatively weak. ITR sites had strong specificity. Seven or eight ITR sites were detected on the arms of chromosomes, not on the ends of chromosomes, making them internal telomere sequences. Two ITR sites were located on the long arm or short arm of chromosomes 1~6, and most of them were located on the long arm of chromosomes 9~16. Certain differences existed in loci intensity and position. Compared with the other seven varieties, 'Yellow Stone' lacked an ITR site, which may be the relatively short length of the telomere sequence in the chromosome, so the site had not been detected. The pachytene chromosomes of this variety can be verified by FISH markers later. At the same time, the coexistence of *Arabidopsis* telomere sequence and other types of telomere sequence on the chromosome of this variety was not excluded. Hyacinth varieties have a long history. The detection of ITR sites on the chromosome arms may caused by multiple structural variations of chromosomes in the long-term evolution of hyacinth, ITR may be formed by the fusion of terminal telomere sequences, or the integration of chromosome segments with telomere sequences into the interior of chromosomes. Compared with that of 45S rDNA and 5S rDNA, the ITR sites intensity was strong. Cox *et al.* (1993) reported that the intensity of

telomere hybridization sites is related to the size of chromosomes. Generally, species with larger chromosomes have stronger hybridization sites of telomere sequences. Therefore, the length of telomere sequences is closely related to the size of chromosomes or genomes. However, the ITR sites in this study were mostly located on shorter chromosomes, in view of the lack of genomic data of hyacinth varieties, we speculated that long telomere-related sequences may exist in some chromosome intervals of hyacinth.

Although rDNA is a highly conserved repeat sequence, its copy number and genetic loci will also change to a certain extent in the process of long-term evolution (Li 2015, Nguyen *et al.* 2016). In addition, hybridization, chromosome structure variation and rearrangement may also lead to changes in rDNA loci and numbers in the genome (Wang *et al.* 2017). The number of rDNA loci in some species, such as *Citrus* (Zhao *et al.* 2016), mostly doubled with ploidy and showed obvious polymorphism and heterozygosity. The loss of DNA repeat loci in the process of species evolution is common, and studies have shown that some tetraploid *Rosa* plants have rDNA locus loss (Tan *et al.* 2019). In this study, the co-localization of 5S rDNA loci and ITR sites in six cultivars was speculated to be caused by chromosome structural variation and chromosome recombination in the process of evolution.

The construction of chromosome FISH maps is an effective method for studying the genetic relationships of species. With the development of FISH technology, multiple DNA repeat probe sets have been successfully used to construct the chromosome physical map of *Sagittaria trifolia* (She *et al.* 2015), *Fragaria vesca* (Qu 2018) and other plants. In this study, the chromosome FISH map of diploid hyacinth was preliminarily constructed by using 45S rDNA, 5S rDNA and telomeric repeats as probes. It was found that 5S rDNA and ITR sites had co-localization and five cultivars loci had isomorphic localization. Therefore, it is still necessary to locate two or even more probes simultaneously to further study the FISH karyotype of diploid hyacinth.

Genetic Relationship and Evolutionary Trend of Diploid Hyacinth:—Eight diploid hyacinth cultivars were divided into three groups by cluster analysis; 'Purple Sensation', 'Blue Pearl' and 'Odysseus' were grouped together in the first category because their ratios of longest to shortest chromosome length were similar. Among the four cultivars, 'Gypsy Queen' had the smallest and the most original asymmetry karyotype coefficient. The karyotype of the three diploid hyacinth cultivars was 2C type in Group II, and 'Yellow Stone' and 'Red Pearl' were grouped together due to the lack of st-type chromosomes. The karyotype of the three diploid hyacinth cultivars was 2C type in Group II, and 'Yellow Stone' and 'Red Pearl' were grouped together due to the lack of st-type chromosomes. 'Gypsy Princess' was in a separate group, Group III. Although its karyotype was 2B type, its asymmetry karyotype coefficient was high.

'Purple Sensation' and 'Blue Pearl' were first clustered into one category in Group I of the cluster analysis. Their genetics were closely related. The karyotype parameters of the two varieties were similar, and the relative length compositions of chromosomes were exactly the same. They were both 2B-type karyotypes and relatively primitive. The chromosome relative length of 'Blue Pearl' varied widely, and the karyotype asymmetry coefficient was high. We speculated that it evolved from 'Purple Sensation' to 'Blue Pearl'. 'Pink Pearl' and 'Red Pearl' were clustered into one category in Group II and were both of the 2C-type karyotype. 'Pink Pearl' had a high karyotype asymmetry coefficient and was composed of m, sm and st chromosomes, while 'Red Pearl' contained only m and sm chromosomes. We speculated that it evolved from 'Red Pearl' to 'Pink Pearl'. 'Gypsy Queen' and 'Odysseus' were both orange varieties, with the 2B-type karyotype and similar karyotype asymmetry coefficients. However, due to the different types of satellite chromosomes, they failed to group together first. Both 'Gypsy Princess' and 'Yellow Stone' were yellow, but they were not clustered together when the euclidean distance was 15. 'Gypsy Princess' was of a 2B-type karyotype, and 'Yellow Stone' was of a 2C-type karyotype. 'Gypsy Princess' has a high nuclear asymmetry coefficient and is composed of m, sm and st chromosomes. 'Yellow Stone' contains only m and sm type chromosomes. It was speculated that the two varieties evolved from different varieties and had the same flower color owing to their similar flavonoid materials and genes controlling flower color formation.

According to Stebbins' theory of karyotype evolution(Stebbins *et al.* 1971), The general trend of higher plant karyotype evolution is from symmetry to asymmetry. Species with older or primitive systematic evolution have more symmetrical karyotypes, while species with higher degree of evolution have low degree of karyotype symmetry and large coefficient of asymmetry. 'Gypsy Princess' had a low average AR value and symmetrical karyotype, which was the most primitive in cluster analysis.The FISH results show that it had four 5S rDNA loci. While three of the other seven cultivars were detected, the above detection phenomenon showed that hyacinth loses 5S rDNA during the evolution process. 'Yellow Stone', 'Red Pearl' and 'Pink Pearl' were clustered in Group I and were closely related to 'Gypsy Princess'. These four cultivars contained an isomorphic location chromosome with one ITR sites on the short arm and a 5S rDNA loci on the long arm. In view of their high degree of karyotype evolution, it can be speculated that chromosome structure variation easily occurs in the evolution of hyacinth. Szinay *et al.* (2012) used a set of bacterial artificial chromosomes (BACs) with specific loci as probes for FISH analysis of *Solanum* crops and determined the

occurrence of inversion in the process of evolution. Lou *et al.* (2013) used a set of chromosome-specific fosmid clones and 45S rDNA probes to perform FISH and found severe recombination suppression of centromeric and heterochromatin domains in cucumber. Therefore, the genetic relationship between hyacinth cultivars and the specific situation of chromosome variation in the process of evolution need to develop novel chromosome-specific markers for further exploration.

Genetic breeding of hyacinth:—Among the original diploid hyacinth cultivars, the red series is dominant, while the blue series only accounts for 15%. Interestingly, the proportion of blue hyacinth is as high as 80% among hyacinth cultivars with more than 29 chromosomes (Hu *et al.* 2011). We conjecture that the chromosome number and structure are related to changes in hyacinth flower color. In this study, 'Gypsy Queen' and 'Odysseus' had the same color and two coincidence loci. 'Gypsy Princess' and 'Yellow Stone' are the same color, and both have 7 ITR sites. However, only diploid cultivars and a few cultivars with the same color were studied in the experiment, and it is still impossible to directly prove that these loci characteristics are related to their flower color. We believe that the specific markers of each chromosome of hyacinth can be further developed to study the genetic relationship and evolution degrees of different hyacinth cultivars. Meanwhile, in-depth study of the source and genome differentiation in polyploid hyacinth is essential to speed up the hyacinth breeding process (Zhao *et al.* 2016).

Polyploidization is a natural phenomenon in the process of plant evolution. Polyploid plants have higher heterozygosity and stronger adaptability to the environment (Ba *et al.* 2016). The chromosome number of horticultural hyacinth varies from diploid to tetraploid, and both polyploid and aneuploid hyacinths are highly fertile (Hu *et al.* 2012). Some researchers believe that the change in rDNA loci is related to genomic polyploidy (Wang *et al.* 2012, Rosato *et al.* 2015). Consequently, hyacinth polyploid breeding research will become an important direction of hyacinth breeding in the future.

By analyzing the chromosomes of polyploid cultivars and hybrid offspring combined with traditional karyotype characteristics, the number and location of the rDNA locus has become one of the reference bases for judging the origin of polyploids, and cytological evidence of its high fertility is expected.

Conclusion

The chromosomes of 8 diploid hyacinth cultivars were studied by FISH and cluster analysis. Eight hyacinth cultivars were divided into three groups by cluster analysis. By comprehensively analyzing the degree of evolution of different cultivars and the distribution of fluorescent loci, we provided valuable clues for determining the genetic relationship between hyacinth cultivars, which can increase the understanding of the evolutionary history and phylogenetic relationships of hyacinth.

List of abbreviations

FISH: fluorescence in situ hybridization; AR: arm ratio; LC: longest chromosome length; SC: shortest chromosome length; ASK: asymmetrical karyotype coefficient; CI: sum of the centromere index; SAT chromosome: satellite chromosome; m chromosome: metacentric chromosome; and sm and st chromosomes: submetacentric and subtelocentric chromosomes.

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