

Chromosome Karyotype Analysis and Evolutionary Trend of Half-Sib Families of Ancient *Ginkgo Biloba* L

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Abstract: The karyotype of 28 families from Shandong, Zhejiang, Guizhou and other 12 provinces exist in half-sib families of ancient *Ginkgo biloba* germplasm repository was studied by squashing method with young leaves from these families. Results: The results showed that the number of chromosomes in 28 families was $2n=2x=24$. The medium-short chromosomes and the medium-long chromosomes were dominant. The karyotype consists of three types: median region chromosome (m), submedian region chromosome (sm) and subterminal region chromosome (st). 71# family is the most primitive, while the 40# family is the most evolved. Families from southwestern China have a low degree of evolution, while those from eastern and southern China have a high degree of evolution.

Keywords: Ancient *Ginkgo* Half-Sib Families, Chromosome, Karyotype and Analysis, Evolutional Trend

1. Introduction

Ginkgo biloba L. is a relict species of Quaternary glaciers, a historical heritage and living fossil, with important economic, ecological and social benefits [1]. *Ginkgo biloba* has a long history of cultivation in China. It flourished in the south of the Yangtze River in the late Han Dynasty, in the Central Plains in the Tang Dynasty and was widely planted in the Yellow River Valley in the Song Dynasty. Although *Ginkgo biloba* is widely distributed in the world, its wild population is only found in China. Studies showed that there are residual wild communities in Southwest China [2-3] and Eastern China [4-5]. As a unique historical and natural landscape, ancient *Ginkgo biloba* is also a precious germplasm resource repository and research material for evolution. In recent years, with the expansion of cities, which leads to the deterioration of ecological environment, the protection of ancient trees is also facing great challenges. Collection, preservation and

evaluation of ancient *Ginkgo biloba* germplasm resources are of great significance for the preservation of precious genes of *Ginkgo biloba*. However, most studies on ancient *Ginkgo biloba* focused on the investigation of germplasm resources and the rejuvenation of weakened ancient trees [6-8], while the exsitu-preservation of ancient *Ginkgo biloba* system is still lacking.

Karyotype analysis is an effective method for discussing plant phylogenetic relationships and phylogenetic evolution. In 1910, Ishikawa [9], a Japanese scholar, reported that the chromosome number of *Ginkgo biloba* was $2n = 24$, which was verified by Pollock [10], Tanaka [11], and Masahiro Hizume [12]. Xing Shiyan et al. [13] analyzed the karyotypes of 21 *Ginkgo biloba* specific germplasms in China, the United States and France, and analyzed the cytogenetics and evolution of *Ginkgo biloba*. Gao Jinhong et al. [14] analyzed the karyotypes of 21 *Ginkgo* fancy breeds and discussed the origin, evolution and classification of *Ginkgo biloba*. Zhang Fang et al. [15]

studied the karyotypes of 28 *Epiphylla* from China and Japan. So far, no studies on karyotype analysis and evolutionary trend of ancient *Ginkgo biloba* have been found. In this study, 28 half-sib families of ancient *Ginkgo biloba* from Shandong, Zhejiang and Guizhou provinces were analyzed by karyotype analysis based on the ancient *Ginkgo biloba* germplasm resource repository established by the research group. The aim is to provide cellular basis for collection, preservation, fine seeds breeding, evolution and cultivation of ancient *Ginkgo biloba* germplasm resources.

2. Materials and Methods

2.1. Materials for Testing

Materials were collected from the half-sib family resource repository of ancient *Ginkgo biloba* in Gaoqiao Forest Farm, Ningyang County. Seeds were sown in April 2014 and a total of 28 families were collected (Table 1).

2.2. Testing Methods

In mid-April 2015, young leaves were collected from 2-year-old seedlings and immersed in the saturated p-dichlorobenzene solution for 8 hours, then fixed with Carnoy's Fluid for 20 hours, dissociated with 1 mol/L hydrochloric acid for 12 minutes, stained with modified

carbol fuchsin for 3 minutes, and squashed into tablets. Chromosomes morphology at metaphase of the cell division was observed under Nikon E200 microscope at 10×40 and 10×100 times and images were obtained.

Chromosome karyotype analysis was performed by two-point four-zone system method of Levan et al. [16], and chromosomes were classified into four groups according to their relative length coefficient by chromosome classification criteria of Kuo et al [17]. Stebbins [18] 's karyotype asymmetry degree was determined according to the ratio of the longest chromosome to the shortest chromosome in karyotype, the proportion of chromosomes with the arm ratio greater than 2, and the karyotype asymmetry coefficient (as.k. C) proposed by Arano [19] on the ratio of the length of the long arm to the length of the whole group of chromosomes.

Chromosome length ratio ($LR = \text{maximum arm length}/\text{minimum arm length}$) was used as the ordinate coordinate, and average arm ratio ($MAR = \text{average long arm}/\text{average short arm}$) was used as the abscissa coordinate to make a two-dimensional evolution trend diagram. $D_{ij}^2 = E_{pk} = (G_{ik} - G_{jk})^2$ formula is used for system analysis. G_{ik} and G_{jk} represent the standardized genotype values of germplasm i, j and the k_{th} trait respectively. SAS 8.2 software was used for Q-type clustering and principal component analysis of 28 families with long arm length, short arm length, LR, MAR and As. K. C factors.

Table 1. Information Table of Seeding Trees of Each Family.

Families No.	Source of ancient trees	Sex	Tree heightt(m)	DBH	Canopy diameter(m×m)	Tree age
6	Tengchong, Yunnan	Female	9.5	1.15	12.0×8.0	300
7	Huishui, Guizhou	Female	30	3.74	10.0×18.0	4000
9	Longkou, ShanDong	Female	23	1.4	20.0×17.0	600
17	Feicheng, ShanDong	Female	16	1.01	13.0×16.0	1000
19	Mengyin, ShanDong	Female	16	0.87	12.0 ×13.0	500
20	Yiyuan, ShanDong	Female	21.5	1.14	16.5×12.5	1300
28	Rushan, ShanDong	Female	21.5	1.14	16.5×12.5	1300
30	Rushan, ShanDong	Female	26.6	2.35	31.0×32.0	1000
40	Lingchuan, Guangxi	Female	30	1.7	24.0×24.0	200
60	Pengshui, Chongqing	Female	22	2	16.0×16.0	250
71	Wudang, Guizhou	Female	21	2.10	-	400
75	Youxi, Fujian	Female	22	0.96	10.5×16.6	500
79	Wanli, Jiangxi	Female	28	2.38	19.5×18.2	1540
82	Rugao, Jiangsu	Female	20.5	1.43	12.0×16.0	1200
86	Songxian, Henan	Female	27	2.9	24.0×28.0	2350
94	Qixia, Shandong	Female	22.5	2.15	34.0×35.0	800
95	Changan, Shaanxi	Female	22	3.31	28.7×28.2	1500
98	Pukou, Jiangsu	Female	20.2	2.42	19.0×15.0	1300
105	Zhucheng, Shandong	Female	24.6	2.73	26.5×25.5	1500
107	Jiaonan, Shandong	Female	22	1.61	21.5×24.5	800
114	Lin'an, Zhejiang	Female	22	1.31	13.0×14.0	210
116	Changshun, Guizhou	Female	35	2.5	25.0×15.0	4000
119	Kangxian, Gansu	Female	37.5	3.21	22.5×22.6	1000
121	Panxian, Guizhou	Female	10	0.60	10.0×12.0	200
123	Huixian, Gansu	Female	23.5	4.82	23.5×24.5	2000
128	Yucheng, Sichuan	Female	30	2.87	18.7×16.6	1000
134	Lingling, Hunnan	Female	30	4.5	30.0×30.0	3000
138	Donggang, Liaoning	Female	26	1.55	-	1300

Note: The collector of ancient ginkgo resources is Xing Shiyang, and the storage location is Gaoqiao Forest, Ningyang.

3. Results

3.1. Chromosome Ploidy in Half-Sib Families of *Ginkgo Biloba*

The results showed that the chromosome numbers of 28 half-sib families of ancient *Ginkgo biloba* were $2n=2x=24$ (Figure 1). The chromosome number and ploidy of *Ginkgo biloba* did not change during the lengthy geological and geochronological changes, which indicated that the chromosome of *Ginkgo biloba* had strong genetic stability.

3.2. Karyotype Analysis of Half-Sib Families of Ancient *Ginkgo Biloba*

Chromosome length ranged from 3.32 to 22.52 μm in 28 half-sib families of ancient *Ginkgo biloba*. The medium-short chromosomes and the medium-long chromosomes were dominant. The karyotype consists of three types: median region chromosome (m), submedian region chromosome (sm) and

subterminal region chromosome (st). The chromosome length ratio of 20 # (Youfang, Zhongzhuang Village, Zhongzhuang Town, Yiyuan County,) family was maximum, which was 3.63. The chromosome length ratio of 82 # (Xiadai Village, Banjing Town, Rugao City, Jiangsu Province) family was minimum, which was 1.59. The average arm ratio of 6 # (Head of household: Sun Zhengyou, Shaba Lijia Xiaozhai, Tengchong County) family was maximum, which was 2.30. The average arm ratio of 128 # (6 Villages and 1 Community, Kongping Township, Yucheng District, Ya'an City, Sichuan Province) family was minimum, which was 1.37. According to Stebbins [16] karyotype symmetry standard, there are 4 types, including 2A, 2B, 3A and 3B. The karyotype asymmetry coefficient of 6# (Head of household: Sun Zhengyou, Shaba Lijia Xiaozhai, Tengchong County) family was maximum, which was 69.74%, and 128# (6 Villages and 1 Community, Kongping Township, Yucheng District, Ya'an City, Sichuan Province) family was minimum, which was 57.83% (Table 2).

Table 2. Major karyotype indicators of 28 ancient *Ginkgo biloba* half-sib families.

Families No.	The range of length/ μm	Length ratio	Mean arm ratio	Karyotype formulae	Relative length form	Karyotype type	As. K. C /%
6	8.38-18.53	2.21	2.30	3m+6sm+3st	2L+2M2+8M1	3B	69.74
7	7.14-14.29	2.00	1.66	6m+5sm+1st	1L+5M2+4M1+2S	2B	62.41
9	7.73-19.42	2.51	2.19	1m+10sm+1st	1L+3M2+8M1	3B	68.62
17	5.54-15.97	2.88	1.85	5m+5sm+2st	1L+5M2+5M1+1S	2B	64.97
19	4.94-15.65	3.17	1.93	4m+7sm+1st	2L+3M2+5M1+2S	2B	65.88
20	3.32-12.05	3.63	1.57	10m+1sm+1st	2L+5M2+2M1+3S	2B	61.06
28	5.73-15.28	2.67	1.74	7m+4sm+1st	1L+4M2+6M1+1S	2B	63.55
30	7.27-20.34	2.80	1.74	9m+2sm+1st	1L+5M2+4M1+2S	2B	63.45
40	6.13-16.31	2.67	2.26	2m+6sm+4st	1L+4M2+5M1+2S	3B	69.36
60	6.18-18.45	2.99	1.71	4m+8sm	1L+4M2+6M1+1S	2B	63.08
71	6.66-12.01	1.80	1.50	8m+4sm	1L+4M2+7M1	2A	60.00
75	5.81-15.66	2.70	1.65	7m+5smt	2L+4M2+5M1+1S	2B	62.23
79	7.39-12.84	1.74	1.86	6m+4sm+2st	3M2+9M1	2A	65.01
82	7.46-11.86	1.59	1.96	2m+9sm+1st	1L+4M2+7M1	2A	66.24
86	6.98-13.47	1.93	1.67	5m+7sm	2L+3M2+6M1+1S	2A	62.49
94	6.17-13.99	2.27	1.93	4m+6sm+2st	1L+4M2+6M1+1S	2B	65.92
95	6.75-14.26	2.11	1.78	5m+7sm	1L+4M2+7M1	2B	64.03
98	3.74- 9.50	2.54	1.59	6m+6sm	2L+3M2+5M1+2S	2B	61.38
105	7.8 -19.01	2.44	1.65	8m+4sm	1L+3M2+8M1	2B	62.33
107	4.05-11.47	2.83	1.80	7m+5sm	2L+3M2+6M1+1S	2B	64.29
114	9.67-18.01	1.86	2.22	3m+6sm+3st	1L+5M2+6M1	3A	68.99
116	3.77-10.61	2.81	1.63	7m+5sm	1L+4M2+6M1+1S	2B	62.01
119	7.19-22.52	3.13	1.75	6m+5sm+1st	1L+2M2+8M1+1S	2B	63.57
121	5.42-12.99	2.40	1.69	6m+6sm	6M2+5M1+1S	2B	62.80
123	7.06-13.78	1.95	1.62	7m+5sm	1L+3M2+8M1	2A	61.86
128	5.27-15.69	2.98	1.37	11m+1sm	2L+1M2+8M1+1S	2B	57.83
134	5.85-13.09	2.24	2.25	3m+5sm+4st	1L+3M2+8M1	3B	69.20
138	8.25-17.00	2.06	1.55	7m+5sm	1L+3M2+7M1+1S	2B	60.84

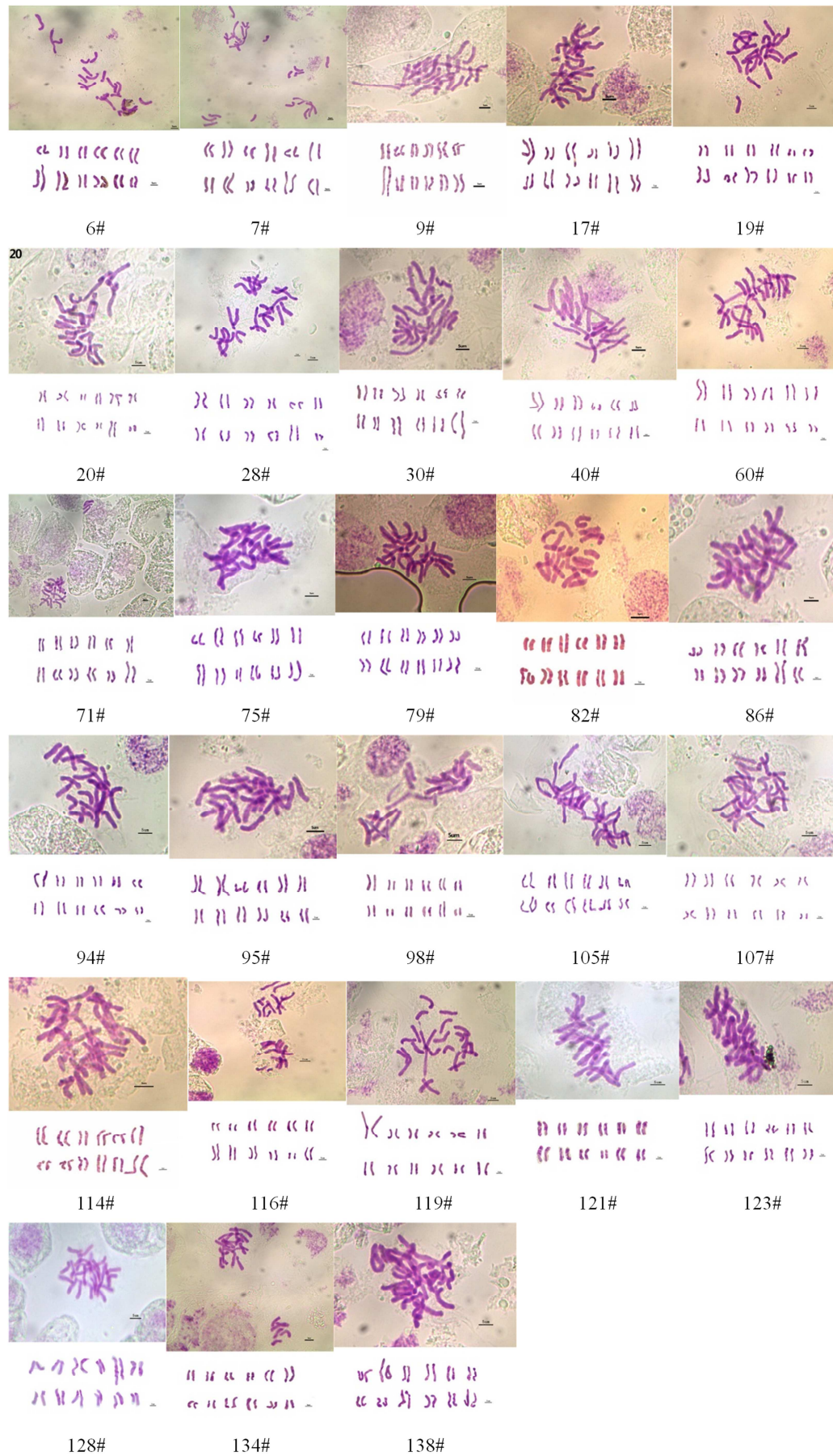
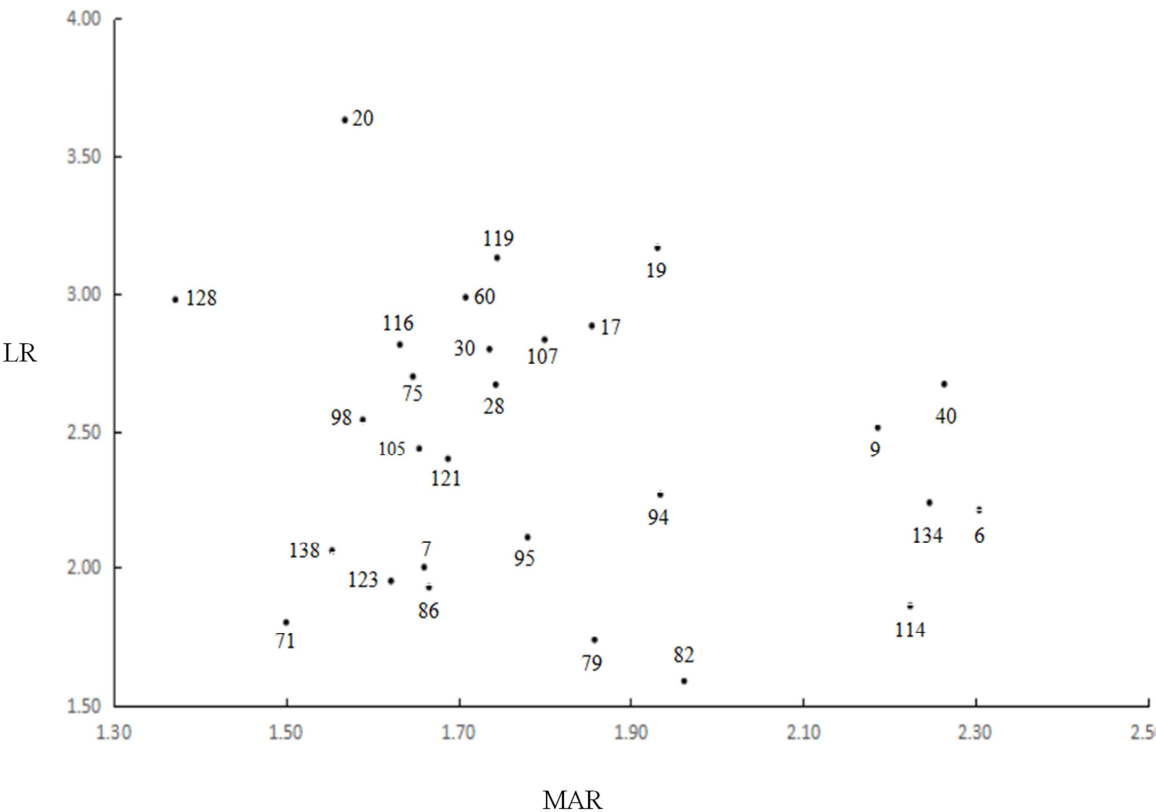


Figure 1. Karyotype and of 28 ancient Ginkgo half-sib families. Families no. are described as Table 1.

3.3. Karyotype Evolution Trend Analysis Of half-Sib Families of Ancient *Ginkgo Biloba*

Stebbins [18] studies showed that karyotype asymmetry was related to some morphological evolution of plants, and the degree of karyotype asymmetry can reflect the degree of plant evolution. The average arm ratio and the length ratio can indicate the asymmetry of karyotypes among different families. The larger these two, the more asymmetric the karyotypes are. From the evolutionary trend map of ancient *Ginkgo biloba* families, it can be seen that the 71#family is the

most primitive at the bottom left of the map, while the 40# family is the most evolved at the top right. The 71 # and 7 # families located in the lower left corner of the map mostly come from Guizhou and Sichuan provinces in southwestern China, and their evolutionary degree is relatively low, while the 6 #, 9 #, 40 #, 134 #families in the upper right corner come from the East and South of China, such as Yunnan and Shandong provinces, and their evolutionary degree is relatively high(Figure 2).



Note: MAR: Arm ratio in mean; LR: Length ratio.

Figure 2. Evolutionary trend map of chromosome karyotype of ancient *Ginkgo biloba* half-sib families.

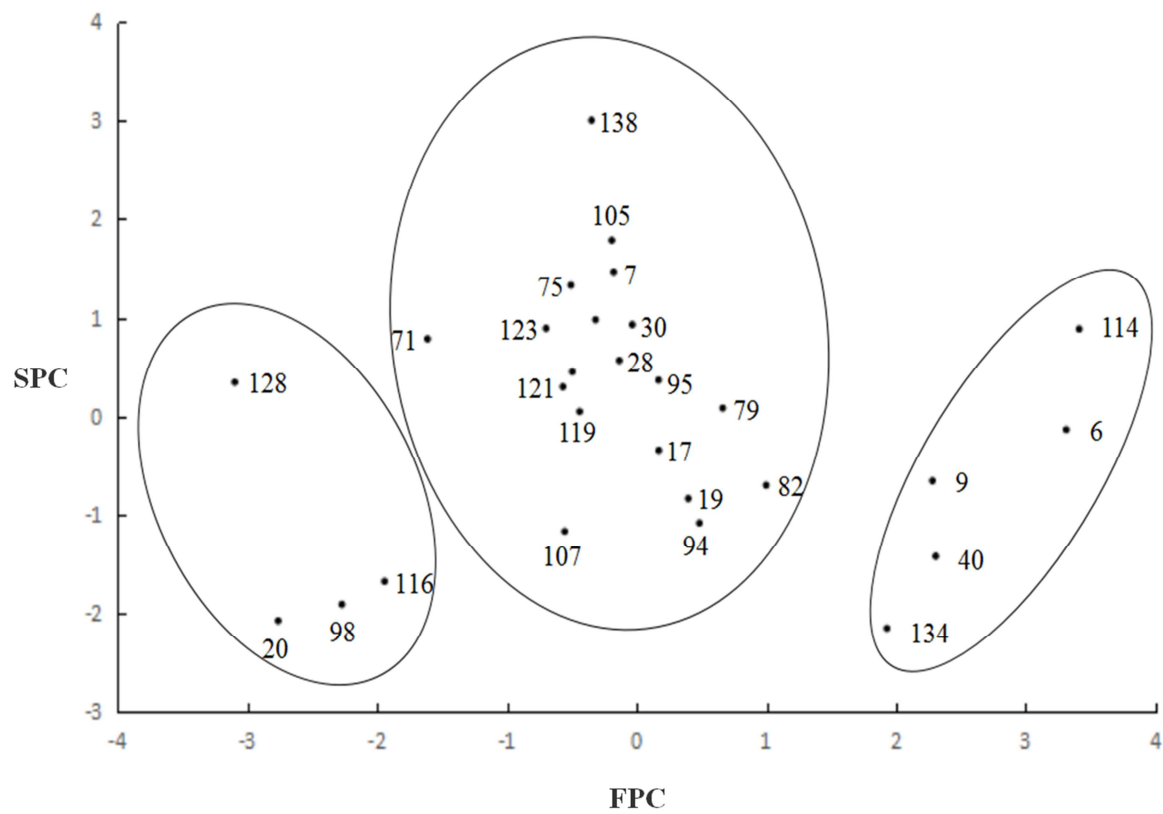
3.4. Principal Component Analysis and Systematic Clustering Analysis

Principal component analysis (PCA) was used to analyze the five factors, including long arm length, short arm length, LR, MAR and As. K. C, by SAS software. The analysis results showed that the contribution ratio of the first principal component was 51.5%, that of the second principal

component was 31.7%, that of the third principal component was 16.6%, that of the fourth and fifth principal components was less than 1%, and the cumulative contribution ratio of the first two principal components was 83.2%. The first two principal components can be selected for analysis, and 28 families can be divided into three categories according to the analysis (Table 3, Figure 3).

Table 3. Eigenvalue and principal components contribution ratio and cumulative contribution ratio.

Character	Eigenvalue	Percentage%	Total percentage%
Long arm	2.5753	51.5056	51.5056
Short arm	1.5848	31.6965	83.2021
Length ratio	0.8297	16.5945	99.7966
Mean arm ratio	0.0065	0.1302	99.9268
As. K. C	0.0037	0.0732	100



Note: F P C: First principal component; S P C: Second principal component

Figure 3. Principal components analysis of ancient *Ginkgo biloba* half-sib families.

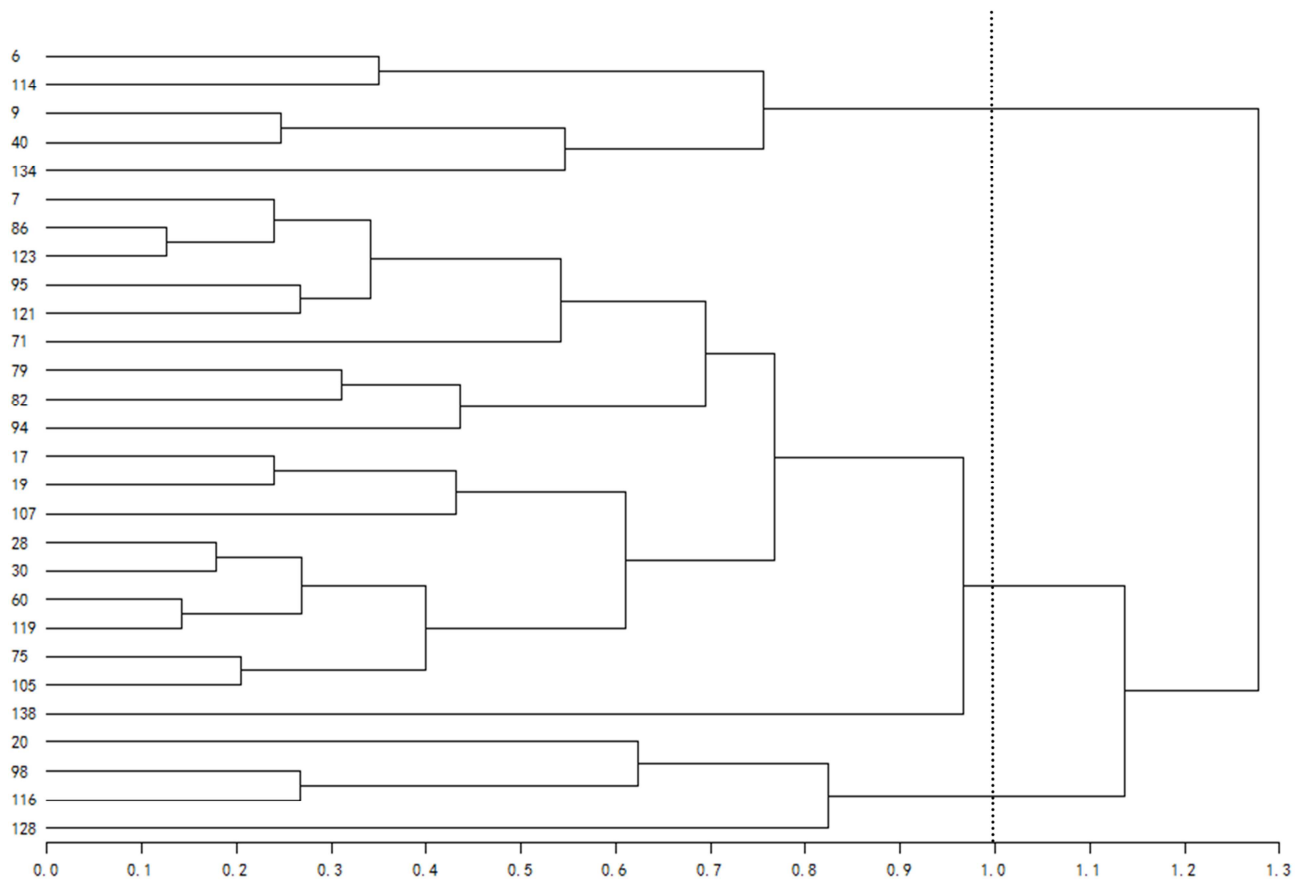


Figure 4. Cluster analysis of ancient *Ginkgo biloba* semi-sib families.

SAS software was used to perform systematic clustering analysis on five factors including long arm length, short arm length, LR, MAR and As-k·C. The analysis results showed that when the threshold value was 1.0, 28 families could be divided into three categories: the I category including 5 families, such as 6 #, 9 #, 40 #, 114 #, 134 #, the II category including 19 families, such as # 7, # 17, # 19, 28 #, 30 #, 60 #, 71 #, 75 #, 79 #, 82 #, 86 #, 94 #, 95 #, 105 #, 107 #, 119 #, 121 #, 123 #, 138 #, the III category including 4 families, such as 20 #, 98 #, 116 #, 128 #. The clustering analysis results were consistent with the results of principal component analysis, and the morphology of chromosomes of the families in the same category was similar (Figure 4).

4. Discussion

The chromosome numbers of 28 half-sib families of ancient *Ginkgo biloba* were $2n=2x=24$, indicating the chromosome number and ploidy of *Ginkgo biloba* did not change during the lengthy geological and geochronological changes, which indicated that the chromosome of *Ginkgo biloba* had strong genetic stability.

Chromosome length ranged from 3.32 to 22.52 μm in 28 half-sib families of ancient *Ginkgo biloba*. The medium-short chromosomes and the medium-long chromosomes were dominant. The karyotype consists of three types: median region chromosome (m), submedian region chromosome (sm) and subterminal region chromosome (st). The chromosome length ratio of 20 # family was maximum, which was 3.63. The chromosome length ratio of 82# family was minimum, which was 1.59. The average arm ratio of 6# family was maximum, which was 2.30. The average arm ratio of 128 # family was minimum, which was 1.37. According to Stebbins [18] karyotype symmetry standard, there are 4 types, including 2A, 2B, 3A and 3B. The karyotype asymmetry coefficient of 6# family was maximum, which was 69.74%, and 128# family was minimum, which was 57.83%. There are great differences in karyotype composition and types among different families of ancient *Ginkgo biloba*, which may be caused by the continuously changing environment in different geographical locations and during the long life history [20], which also provides abundant resources for the selection and preservation of fine families of ancient *Ginkgo biloba*.

In terms of evolutionary trends, 71# family is the most primitive and 40# family is the most evolved. In trend map, 71# and 128 # families at the lower left corner of the coordinate system come from southwest China, which is relatively primitive. In addition, the fact that the families of southwestern provinces such as Guizhou and Yunnan provinces are clustered together with those of Eastern Jiangsu and Zhejiang provinces shows that the ancient Ginkgo biloba in the two regions have relatively close genetic relationship, which is consistent with the results of Gong Wei [21] speculation that the southwestern region and the western

Tianmushan region of eastern Zhejiang Province are two refuges of Ginkgo biloba in China during the Quaternary glacial period. Families such as 6#, 9#, 40# and 134# are located at the upper right corner of the trend chart. These families all come from eastern and southern regions and have a high degree of evolution.

The chromosomes of the families from Shandong and Yunnan regions were clustered into one category except for 20# family, which indicated that the similarity of chromosomes was high, the genetic traits were stable, and the evolutionary degree of the families from the two regions was high. Therefore, Shandong and Yunnan provinces could be taken as good provenance selection areas for ancient *Ginkgo biloba*. However, the 20# family was specific germplasm of *Epiphylla*, whose karyotype was similar to families with low evolutionary degree such as 116# and 128# families. The clustering results also showed that the chromosome morphology of 20# family was similar to those of 116# and 128# families, which was relatively primitive, and may be related to the atavism of *Epiphylla*.

5. Conclusion

The chromosome numbers of 28 ancient ginkgo half-sib families were $2n=2x=24$, and the chromosome length ranged from 3.32 μm to 22.52 μm . The medium-short chromosomes and the medium-long chromosomes were dominant. The karyotype consists of three types: median region chromosome (m), submedian region chromosome (sm) and subterminal region chromosome (st). The karyotype asymmetry coefficient of 6# family was maximum, which was 69.74%, and 128# family was minimum, which was 57.83%.

From the perspective of evolutionary trends, ginkgo evolved from the southwest to the east and south of China. The family in the southwestern part of China was relatively primitive, while the families from the east and south were more evolved. 20# family was specific germplasm of *Epiphylla*, whose karyotype was similar to families with low evolutionary degree such as 116# and 128# families, which was relatively primitive, and may be related to the atavism of *Epiphylla*.

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