Phylogenetic Relationship of Lilies (Lilium) Analyzed based on trnH-psbA Barcode Technology

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Abstract Phylogenetic relationship of lilium plants was studied by means of trnH-psbA DNA barcode technique. The result demonstrated that trnH-psbA sequence length varied in different lilium plants. The entire length of trnH-psbA sequence was 460 bp after multiple alignments. The number of conserved sites was 415 and that of variable sites was 45. A total of 63 tested materials were clustered into five groups. Lilium davidii, 7 Asiatic cultivars, one cross progeny between Asiatic cultivar and wild parental species, were clustered into one subgroup (the I a subgroups). Lilium speciosum var. gloriosoides, 10 Oriental cultivars and 2 extra-section Archelirion cross progenies were gathered into one subgroup (the I b subgroup ). Majority of the tested wild species were clustered into the II group which showed the characteristics of intra-species and geography. To some extent, the phylogenetic relationship of wild lilies in II group via trnH-psbA barcode was inconsistent with the result obtained from traditional morphological classification method. The other 4 Asiatic cultivars and one cross progeny between one Asiatic cultivar and wild parental species were clustered into the III group. Therefore, the Asiatic cultivars in I b subgroup and those in the III group were originated from different lilies in the section Sinomartagon.

Keywords Lilium brownie, Phylogenetic relationship, trnH-psbA barcode

Introduction

Lily has an unusual richness of germplasm resources which about 8 000 cultivars have been registered (Gu, 2013). To study their phylogenetic relationship could be helpful to reveal the relationship among interspecies, intraspecies and cultivars; to find new species or unclear (obscure) species would facilitate to building the basis for parent selection in hybrid breeding program as well. Morphological markers (Li, 2010) and cytological markers (Peng, 2012) were used to mine the phylogenetic relationship among species, yet phenotypic diversity is not always able to present the real diversity in essence of hereditary because the morphological characteristics should be the results generated by combined actions between genetic effect and external environment factors. The molecular genetic markers are rarely used in the research of lily phylogenetic relationship due to the limited numbers of the markers. In recent years, RAPD (Li, 2013), SRAP (Zhen, 2010), SSR (Yu, 2013), ISSR (Wu, 2010) and other molecular markers have been used to study the phylogenetic relationship of lilies. RAPD amplification is poor reproducible due to its short length of primer; SRAP is distributed randomly and irregularly on the chromosome due to the molecular marker failed to use of the amplification sequence, and the marker is applied with high cost; screening and identifying the SRAP needs heavy workload, but the efficiency is pretty low as well. The amplification by ISSR has better stability that of RAPD due to its longer length of primer. The three kinds of molecular markers mentioned above would be able to achieve the good results in analysis of intraspecific relationship, but limitations applied in analysis of interspecies.

DNA barcoding is a molecular biological technique using DNA sequence information to identify species or their variant types. It mainly uses a group of short homologous DNA sequences coming from different biological
individuals to carry out multiple sequence alignment and clustering analysis, and then merges an individual into a certain taxonomical group accurately. The following short homologous DNA sequences were commonly applied in the plant barcoding: code region fragment of chloroplast genome such as rpoB (subunit of RNA polymerase b), rpoC1 (ribonucleic acid polymerase C1 subunit), matK (maturase K protein), rbcL (1,5 diphosphate ribulose-1,5-bisphosphate carboxylase) and non-coding region fragment such as trnH psbA (plastid trnH psbA spacer), fragment of the nuclear genome ITS (ribosomal internal transcribed spacer region) (Yan and Yu, 2010). TrnH-psbA sequence is a fragment of the chloroplast space (non-coding region), and its two sides contain a conservative sequence of about 75 bp, which can be used to design universal primers, yet this sequence has a high frequency of insertion and deletion; besides, the length differences of amplification among different species are significant, so that this homologous DNA sequences has a good recognition for the allied species (Lahaye et al., 2008). But so far we are unable to find any report about the application of DNA barcoding technology based on trnH-psbA sequence to analyze the phylogenetic relationship among lily systems. In order to provide a theoretical reference for the combinations of lily crossbreeding, we used trnH-psbA barcode technology to study the phylogenetic relationship among 67 lily germplasms, as well as to determine its fitness on interspecies and intraspecies classification in this research.

1 Results and Analysis

1.1 Electrophorogram of lilies amplified based on trnH-psbA gene sequence
Amplified products were detected by 1% agarose gel electrophoresis. The results showed that this primer could reach 100% amplification efficiency (Figure 1) and generate clear and stable fragments with the sizes ranged from 400 bp to 500 bp in length.

![Figure 1 Agaros electrophorogram of lily in part based on trnH-psbA gene sequence](image)

Note: M: DNA ladder marker 1500

1.2 Sequence alignment of lily based on trnH-psbA gene sequence
Homologous alignment of the sequencing results of the trnH-psbA barcode in 67 samples were carried out by DANMAN. The results indicated that there be certainly differences in the length of trnH-psbA sequence in lilies. Total sequence length is 460 bp after sequence alignment, which implied that there be happens of insertion, substitution and deletion obviously. There were 415 conserved sites and 45 variable sites detected in this research.

1.3 Construction of phylogenetic tree of lilies based on chloroplast trnH-psbA gene sequence
The phylogenetic tree of 67 lily samples was constructed by PHYLIP. Figure 2 showed that Yebaihe NO.28 (Lilium brownii) (germplasm origin from Lichuan Baiyang Town) and Yebaihe NO.62 (‘White Heaven’×Lilium bakerianum var. delavayi) first formed a new branch and were classified into a group respectively from the bottom of the tree. Then, Baihua Baihe NO.30 (Lilium brownii var. viridulum) was also divided into a group and formed a single branch, and Chuanbaihe NO.25 (Lilium davidii) (germplasm origin from Yulong Snow Mountain) was separated as well, indicating that the above 4 lily samples had farther relationship with the rest 63 samples.

And then, we classified the rest 63 tested samples into five groups. The 24 samples on the top were classified into the first category (Category 1), which mainly consist of cultivated sample, and Category 1 can be further divided into 2 subgroup (Ia and Ib). Subgroup Ia contained 10 lily samples, including Chuanbaihe No.33 (L. davidii) (germplasm origin from Xizhou Town, Dali City) and Chuanbaihe No.34 (L. davidii) (germplasm origin from Yulong xueshan, Lijiang City), both of them were the primitive parents of Asian lily (Lilium asiatic). Hybrid offspring No 65 of Pollyanna’×Lilium dauricum came from the cross of Asian lily (L. asiatic) and Maobaohe (L.
dauricum), one of the wild parent of Asian lily (Lilium asiatic), and the rest 7 samples were all the Asian lilies (Lilium asiatic). ‘Avignon’ No.67, ‘Tinos’ No.41, ‘Matrix’ No.43, ‘pollyanna’×Lilium dauricum No.65 and ‘Landini’ No.39 converged into one unit, and Chuanbaihe No.33 (L. davidii) (germplasm origin from Xizhou Town, Dali City) and Chuangbahei No.34 (L. davidii) (germplasm origin from Yulong xueshan, Lijiang City) converged into another. These two units combined into one phylogenetic group, which then combined with the unit consisting of ‘Red latin’ No.57, ‘Detroit’ No.60 and ‘Sorpressa’ No.46, indicating that they all have a close intraspecific relationship. In summary, subgroup I a was composed of the primitive parents of Asian lily (such as Chuanbaihe), Asian lily and it's hybrid offspring of wild parent, and their genetic relationship were close.

Figure 2 Phylogenetic tree of lily plants clustered based on trnH-psbA sequences

Note: 1: Lilium brownii var. viridulum; 2: Lilium brownie; 3: Lilium brownie; 4: Lilium tigrinum; 5: Lilium tigrinum; 6: Lilium tigrinum; 7: Lilium tigrinum; 8: Lilium tigrinum; 9: Lilium tigrinum; 10: Lilium leichtlinii var.maximowiczii; 11: Lilium leichtlinii var.maximowiczii; 12: Lilium sulphureum; 13: Lilium sulphureum; 14: Lilium bakerianum var. aureum; 15: Lilium dauricum; 16: Lilium dauricum; 17: Lilium dauricum; 18: Lilium concolor var. megalanthus; 19: Lilium leucanthum; 20: Lilium henryi; 21: Lilium duchartrei; 22: Lilium taliense; 23: Lilium bakerianum var.delavayi; 24: Lilium bakerianum var.dwarf; 25: Lilium davidii; 26: Lilium distichum; 27: L. distichum var. odorata; 28: Lilium brownie; 29: Lilium pumilum; 30: Lilium brownii var.; 31: Lilium tigrinum; 32: Lilium tigrinum; 33: Lilium davidii; 34:Lilium davidii; 35: Lilium speciosum var. gloriosoides; 36: Tresor; 37: Apricot pixels; 38: Yellow pixels; 39: Landini; 40: Lolly pop; 41: Tinos; 42: Loreto; 43: Matrix; 44: Gironde; 45: White plexs; 46: Sorpressa; 47: Constanta; 48: Canberra; 49: Corvara; 50: Tiber; 51: Francia; 52: Caruso; 53: Mona lisa; 54: Energetic; 55: Sorbonne; 56: Allstar; 57: Redlatin; 58: Renoir; 59: New; 60: Detroit; 61: New; 62: White Heaven ×Lilium bakerianum var.delavayi; 63: Tiber×Sorbonne; 64: NavonaxLilium dauricum; 65: Pollyanna×Lilium dauricum; 66: SorbonnexLilium speciosum var. gloriosoides; 67: Avignon
Subgroup I b contained 14 lily samples. Asian lily ‘Tresor’ No.36 and Meiyanbaihe No.35 (germplasm origin from Yongkang City, Zhejiang Province) formed a unit respectively. For the rest 12 samples, hybrid offspring ‘Sorbonne’×Lilium speciosum var. gloriosoides No.66 came from the cross of Oriental lily and wild lily that belongs to section archelirion, hybrid offspring ‘Tiber’×‘Sorbonne’ No.63 came from the cross of Oriental lily varieties, and the rest 10 samples were all Oriental lilies. 7 samples including ‘Energetic’ No.54, ‘Sorbonne’×Lilium speciosum No.66, ‘Sorbonne’ No.55, ‘Canberra’ No.48, ‘Caruso’ No.52, ‘Tiber’×‘Sorbonne’ No.63 and ‘Allstar’ No.56 were combined into one unit, which combined with the unit of 5 samples consisting of ‘Mona lisa’ No.53, ‘Corvara’ No.49, ‘Tiber’ No.50, ‘Francia’ No.51 and ‘Constanta’ No.47 into a phylogenetic group, along with the units of ‘Tresor’ No.36 and Meiyanbaihe (Lilium speciosum var. gloriosoides) No.35, clustered together in the subgroup I b. In the subgroup I b, except Asian lily ‘Tresor’ No.36, the rest 13 germplasms consisted of wild lily that belongs to section archelirion (the primitive parent of oriental lily), oriental lily and hybrid offspring came from the group whose female parent is oriental lily, and their genetic relationship were close.

The 24 lily samples in the middle part of the phylogenetic tree were classified as the second category (Category II), which mainly composed of wild lilies as well as a small amount of cultivated lilies. Category II could be divided into 3 subgroups. The first subgroup (subgroup II a) contained 16 lily samples with quite complex types, which were wild lilies except those four Asian lilies called ‘Yellow pixels’ No.38, ‘Apricot pixels’ No.37, ‘White pixels’ No.35 and ‘Loreto’ No.42. Subgroup II was divided into 4 branches, and the first branch (Branch II a-1) contained one material which was called Dahua baihe No.18 (L. megalanthum) (germplasm origin from Antu Yongpingxiang, section of Sinomartagon). Nine lily materials were contained in the second branch (Branch II a-1), they were Juandan No.31 (L. lancifolium) (germplasm origin from Shanxi Qinling, section of Sinomartagon), Juandan No.5 (L. lancifolium) (germplasm origin from Foping County, Longcaoping Village, section of Sinomartagon), Juandan No.6 (L. lancifolium) (germplasm origin from Changbai Moutain, Erdaobaibaihe Town, section of Sinomartagon), Shandan No.29 (L. Pumilum) (germplasm origin from Foping County, Longcaoping Village, section of Sinomartagon), wild lily No.2 (germplasm origin from Badong County, Chadianzi Town, section of Leucolirion), Juandan No.9 (L. Lancifolium) (germplasm origin from Taibai County, Taochuan Town, Baiyangyuan Village, section of Sinomartagon), Danhuanghua baihe No.13 (L. sulphureum) (germplasm origin from Yunnan, Eryuan County, Sanying Town, section of Leucolirion), ‘Yellow pixels’ No.38 (asiatice hybrids) and Juandan No.4 (L. lancifolium) (germplasm origin from Shenlongjia Area, Muyu Town, Shicaoho Village, section of Sinomartagon), in which 5 materials were produced in different regions of L. lancifolium. The third branch (Branch II a-3) contained Juandan No.7 (L. lancifolium) (germplasm origin from Antu County, Yongqing Village, section of Sinomartagon) and ‘Apricot pixels’ No.37 (asiatice hybrids). 4 materials were contained in the fourth subfield (Subfield II a-4), they were ‘White pixels’ No.45, Baoxing baihe No.21 (L. duchartrei) (germplasm origin from Heqing County, Niutou Mountain, section of Sinomartagon), ‘Loreto’ No.42 and Yichang baihe No.19 (L. Leucanthum) (germplasm origin from Badong County, Chadianzi Town, section of Leucolirion). 3 lily materials were contained in the second subgroup (Subgroup II b), they were Lunye baihe No.26 (L. distichum) (germplasm origin from Antu County, Yongqing Village, section of Martagon), Maboaihe No.15 (L. dauricum) (germplasm origin from Antu County, Yaotuan forest farm, section of Daurolirion), Dahua juandan No.11 (L. leichtlinii ) (germplasm origin from Tonghua City, Zuoan Villag, section of Sinomartagon), all 3 samples were the source of the Northeast Jilin. 5 lily materials were contained in the third subgroup (Subgroup II c), they were Dahua juandan No.10 (L. leichtlinii) (germplasm origin from Jilin, section of Sinomartagon), Maboaihe No.17 (L. dauricum) (germplasm origin from Changbai Moutain, Erdaobaibaihe Town, section of Daurolirion), Maboaihe No.16 (L. dauricum) (germplasm origin from Changbai Moutain, Toudao Station, germplasm origin from section of Sinomartagon), ‘Lolly pop’ No.40 (asiatice hybrids), Lunye baihe (L. distichum) (germplasm origin from Antu County, Yaotuan forest farm, section of Martagon). The remaining 4 materials were the wild lily species originating from Northeast Jilin, except the ‘Lolly pop’ No.40 (asiatice hybrids). Subgroup II b and II c lined much closer in the system tree, which suggesting its close relationship. Most materials of these two
Five lily samples below the second category in the phylogenetic tree were classified as the third category (Category III) class (III), ‘Gironde’ No.44, ‘Val di sole’ No.59, ‘Madras’ No.61, ‘Navona’ × Lilium dauricum No.64 and ‘Renoir’ No.58. Among these five samples, four of them were Asian lilies except (‘Navona’ × Lilium dauricum) No.64 was hybrid offspring derived from Asian lily and one of its primitive parents.

The two lily materials were clustered into the fourth class (IV) below the third class in the clustering figure, they were No.20 Hubei Baihe (Xuanen county Wanzhai township, section sinomatagon), No.3 Yebaihe (Shenlongjia songluo town, Section Leucolirion), both of them were coming from Hubei province.

On the bottom of phylogenetic tree, there were 8 wild lilies, clustering into the fifth classes (Class V), which can be further divided into 2 subclasses. The first subclass (Va) was composed by four samples: No.12 Danhuabaite (Shenlongjia Hongping town, section leucolirion), No.1 Lily (Xuanen county Wanzhai township, section leucolirion), No.32 Juandan (Taibai county Taochuan town, Sect. Sinomartagon), and No.8 Juandan (Fengchen county Baoshan town, Sect. Sinomartagon), which includes both section lilium and section sinomatagon (e.g. No.32 and No.8).

The second subclass (Vb) all came from Northwest Yunnan, including No.23 Huanglv huidian (Dali city Cangshan wutaifeng, generalized section sinomatagon), No.22 Dalibaihe (Binhuan county Laishan mountain, section sinomatagon), No.24 Dianbaihe (Lijiang Yulongxueshan mountain, generalized section sinomatagon), and No.14 Huangjinhua Dianbaihe (Haidong county Daqingshan mountain, generalized section sinomatagon).

2 Discussion

2.1 Comparison between traditional classification method and the trnH-psbA barcode technology in lily phylogenetics

When we use traditional morphological classification method to do the phylogenetic classification for original Lily species, flower type is the first classification criteria in Chinese morphological taxonomy method. They will be divided into four groups: section lilium, section lophophorum, section sinomatagon and section martagon. This classification method will merge some wild original parents of Asian cultivar (such as L. speciosum and its variants L. speciosum var. gloriosoides) into Section sinomatagon, and couldn’t explain the fact of hybrid difficulty and distant genetic relationship between Asian cultivar and Asian Lily (originated in Section sinomatagon). While the national morphological taxonomy usually take leaf type as the first classification criteria, flower type as the second classification criteria, combined with epigaeous, bulb color and shape divided original Lily species into seven groups. The L. speciosum, L. speciosum var. gloriosoides are segmented into section archelirion instead of section sinomatagon which provide a well explaination for the fact of distant genetic relationship between Oriental cultivar and Asiatic Lily. Therefore, there obviously are limitations in Chinese traditional morphological methods in Lily classification, and the classification of Lily into seven groups according international system is now generally used.

In our study, the second category (II) included 4 sections of wild lilies, i.e. section of Sinomartagon, section of Leucolirion, section of Daurolirion and section of Martagon. Although Danhuanghua Bahe (Lilium sulphureum)(germplasm origin from Sanying Town, Eryuan County) and Yichang Bahe (Lilium leucanthum) (germplasm origin from Chadianzi, Badong County) should be grouped in the section of Leucolirion, the evidence presented that they can’t get together, which might be due to these wild lilies came from different geographic regions and different ecological environments where exist much more more geographical isolation and reproductive barrier, resulting in the evolution of the chloroplast genes along different ways.

In addition, in the fourth category (Category IV), Hubei Baihe (Lilium henryi) belongs to section of Sinomartagon and wild lilies belongs to section of Leucolirion. According to the traditional taxonomic to analysis hybridization between the two groups is very difficult. But it has been reported that Hubei Baihe (Lilium henryi)
could be hybridized with wild lilies, anhuanghua Bahe (Lilium sulphureum), Ziji Baihe (Lilium leucanthum), Meili Baihe (Lilium speciosum Thunb), etc, which gain a lot of famous hybrids (Hu Bingfen, 2003). So, the phylogenetic relationship of Hubei Baihe (Lilium henryi) and subgroup of Leucolirion are closer. Hubei Baihe (Lilium henryi) and wild lilies together as a category also shows that trnH-psbA Barcode classification can be complementary with the traditional taxonomy. At present, trnH-psbA Barcode classification has become a mainstream technology used in taxonomy. Quan Miaohua et al. effectively identification and classification of *Lycoris* using chloroplast trnH-psbA gene sequence to analyzed the relationship between the 16 kinds of *Lycoris* (Quan Miaohua, 2011). John etc., using Barcode technology analyzed the system relationships of 48 genera 96 species of plants, found that the correct recognition rate of trnH-psbA single Barcode is 83%, the correct recognition rate of rbcL-a+trnH-psbA Combined Barcode up to 95%. Using a Barcode to reflect the system relationship may have some limitations, but the combined Barcode can overcome this limitation to a certain extent (John et al., 2005).

### 2.2 Comparison of classification between wild lilies and cultivated lilies

From the results of the phylogenetic clustering, it could be seen that the cultivated lilies of Asian lilies basically grouped into teo sub-categories Asian lily (Ⅰ a) and Oriental lily (Ⅰ b). Asian hybrids lily also named Chaotian ilium, which hybrid were produced by lily species with distributed in Asian area. The parents of Asian hybrids lily included Lilium amabile (*L. amabile*), Lilium bulbiferum (*L. bulbiferum*), Lilium concolor (*L. concolor*), Lilium dauricum Ker-Gawl (*L. dauricum*). David lily (*L. davidii*) at least 12 species. L. davidii is one of the original parents of Asian lilies, Asian lily (*L. davidii*), and Sect. Sinomartagon Comber hybridized a subclass which was consistent with close relationship. Oriental lily mainly hybridized by petiolate group (Section *Archelirion*) with several wild lilies, and which parents included safflower lily, Lilium auratum (*L. auratum*), Japanese lily, Lilium speciosum (*L. speciosum*) and Hubei Lily (*L. henryi*). Lilium speciosum (*L. speciosum* var. *gloriosoides*) is variant of Lilium speciosum, and is the original parents of Oriental lily. The subclass was hybridized by oriental lily, *L. speciosum* var. *gloriosoides* and petiole group, which relationship was consistent with *L. speciosum* var. *gloriosoides*.

Most of wild lilies cluster in the second category (category Ⅱ ), the forth category (category Ⅳ ) and the fifth category (category Ⅴ ), which exhibits the feature of Intraspecific assembling and geographical aggregation. For example, the five materials of *Lilium lancifolium Thunb* were collected from different province and group, but which all were gathered in the Ⅰ a sub class’ 2nd branch, which also reflected the close genetic relationship within Intraspecific different group. II B subtypes and II C subclass contained a total of 8 materials, except the one Asian lilies, the other 7 materials were collected from Jilin Changbai mountain area. Those 7 materials were belonged to Sect. Sinomartagon Comber, Sect. Martagon Rchb and *Lilium dauricum* group and so on. For example, the 4 materials of V b subclass were collected from Yunnan Lijiang and Dali area, which of 3 materials were gathered by the variant of *Lilium bakerianum Coll. et Hems*. The same area’s Lilium plants might be happened some genetic exchange due to the long-term close contact.

Besides, Cultivated lilies and wild lilies (Lilium brownii) were gathered together in the first Category (Category Ⅰ ), which indicated that the genetic relationship between them were close, and there are able to compose of hybrid combinations as well as took a theory basis for prediction the hybridization affinity. For example, the Ⅰ c subgroup 'Lolly pop' and 'White pixels' growth in Aisa with peculiar color clustered together with Baoxing lily (*L.uchartrei* germplasm origin from Niuotou Mountain, Heqing County) the Fragrance variant of lunye lily (*L.medeloides*) found in nature, which indicated their genetic relationship are close, and there are able to consist of hybrid combinations as well as make it possible to cultivate the fragrance of Aisan lily (*Lilium Asiatica Hybrida*). In addition, they are also prospective in lies market, such as ‘YYellow pixels’ × *liliumlancifolium*, ‘Apricot pixels’ × *liliumlancifolium*, Loreto × *Yichang lily* (*L.leucanthemum*)

### 2.3 Conclusion

In summary of this thesis, the Lilium trnH-psbA sequences of 67 germplasm resources were compared and
constructed phylogenetic tree by using the barcode technology, the different of The Lilium trnH-psbA sequences of 67 germplasm resources were in-depth analysed as well. Which analyzed the position of 35 wild lilies (Lilium brownii) and 32 cultivars. Also provided a theoretical basis and reference for taxonomic research and hybrids breeding research between wild lily (Lilium brownii) and the cultivated, so that researchers can develop, utilize and innovate to the wild lily (Lilium brownii germplasm) resources scientifically and effectively.

3 Materials and Methods

3.1 Experimental materials

Thirty-five wild lilies and thirty-two cultivars as experimental materials (Table 1) were used to extract their DNAs from the leaves of tissue culture seedlings

Table 1 The list of lilies used for analysis of trnH-psbA DNA barcode

| No. | Variety name | Origin | Type | No. | Variety name | Type |
|-----|--------------|--------|------|-----|--------------|------|
| 1   | Lilium brownii var.viridulum | Xuanen county Wanzhai township, Hubei | 36 | Tresor | A |
| 2   | Lilium brownii | Chadianzi town, Hubei | 37 | Apricot pixels | A |
| 3   | Lilium brownii | Shennongjia songliao town, Hubei | 38 | Yellow pixels | A |
| 4   | Lilium tigrinum | Shennongjia muiyu shuacho township, Hubei | 39 | Landini | A |
| 5   | Lilium tigrinum | Foping county Longcaoqing township, Shanxi | 40 | Lolly pop | A |
| 6   | Lilium tigrinum | Changaishan city Erdaohei town, Jilin | 41 | Tinos | A |
| 7   | Lilium tigrinum | Antu county Yongqing township, Jilin | 42 | Loreto | A |
| 8   | Lilium tigrinum | Fengchen county Baoshan town, Jilin | 43 | Matrix | A |
| 9   | Lilium tigrinum | Taibai county Baiyangyuan township, Shanxi | 44 | Giroonde | A |
| 10  | Lilium leichtlinii var.maximowiczii | Changaishan mountain, Jilin | 45 | White pixels | A |
| 11  | Lilium leichtlinii var.maximowiczii | Tonghua city Zuoan township, Jilin | 46 | Sperianssa | A |
| 12  | Lilium sulphureum | Shennongjia Hongqing town, Hubai | 47 | Constanta | O |
| 13  | Lilium sulphureum | Eryuan county Sanyan town, Yunnan | 48 | Canberra | O |
| 14  | Lilium bakerianum var. aureum | Haidong county Daqingshan mountain, Yunnan | 49 | Corvara | O |
| 15  | Lilium dauricum | Antu county Yaotan forestry, Jinlin | 50 | Tiber | O |
| 16  | Lilium dauricum | Changaishan Toubaotai town, Jilin | 51 | Francia | O |
| 17  | Lilium dauricum | Changaishan Erdaohei town, Jilin | 52 | Caruso | O |
| 18  | Lilium concolor var.megalanthum | Antu county Yongquin township, Jilin | 53 | Mona lisa | O |
| 19  | Lilium leucanthum | Badong county Chadianzi town, Hubei | 54 | Energetic | O |
| 20  | Lilium henryi | Xuanen county Wanzhai township, Hubei | 55 | Sorbonne | O |
| 21  | Lilium duchartrei | Heqing county Niutoushan mountain, Yunnan | 56 | Allstar | O |
| 22  | Lilium thaiense | Binchuan county Laishan mountain, Yunnan | 57 | Redlatine | A |
| 23  | Lilium bakerianum var.delavayi | Dali city Cangshan wutaifeng, Yunnan | 58 | Renoir | A |
| 24  | Lilium bakerianum var.dwarf | Lijiang Yulongxueshan mountain, Yunnan | 59 | New 59 | A |
| 25  | Lilium davidii | Lijiang Yulongxueshan mountain, Yunnan | 60 | Detroit | A |
| 26  | Lilium distichum | Antu Yongqing township, Jilin | 61 | New 61 | A |
| 27  | L.distichum var.odorata | Antu county Yaotan forestry, Jinlin | 62 | White Heaven ×LiliumCP | |
| 28  | Lilium brownii | Changaishan mountain, Hubei | 63 | Tiber×Sorbonne | CP |
| 29  | Lilium pumilum | Foping county Longcaoqing township, Shanxi | 64 | Navona×Lilium dauricum | CP |
| 30  | Lilium brownii var.vindulm | Foping county Longcaoqing township, Shanxi | 65 | Pollyanna×Lilium dauricum | CP |
| 31  | Lilium tigrinum | Qinlin mountain, Shanxi | 66 | Sorbonne×Lilium speciosumCP | CP |
| 32  | Lilium tigrinum | Taibai county Taochuan town, Shanxi | 67 | Avignon | CP |
| 33  | Lilium davidii | Dali city Xizhou town, Yunnan |  |  |
| 34  | Lilium davidii | Lijiang Yulongxueshan mountain, Yunnan |  |  |
| 35  | Lilium speciosum var.gloriosoides | Yongkang city, Zhejiang |  |  |

Note: A : Asiatic cultivar; O: Oriental cultivar ; CP: Cross progenies

3.2 Experimental methods
3.2.1 Total DNA extraction
SDS method was used to extract the total DNA of the experimental materials, the concentration of DNA was detected by 1% agarose gel electrophoresis (Figure 1).

3.2.2 Amplification of chloroplast trnH-psbA gene
According to reference documentation (Fazekas et al., 2008), we selected universal primers for the amplification of chloroplast trnH-psbA gene. The upstream and downstream primer sequences are F: 5’-CGCGCATGGTGGAATTCAACA-3’, R: 5’-GTTATGCGTAACGTAATGCTC-3’. PCR amplification system was as follows: 10× Buffer 2.5 μL, dNTPs 2.5 μL, upstream and downstream primers were 0.5 μL, LA Taq enzyme0.25 μL, template DNA 5 μL, ddH2O 14.25 μL, and total volume 25 μL. PCR reaction conditions were as follows: 94℃ pre denaturation 5 min, 94℃ denaturation 30s, 56℃ annealing 30s, 72℃ extending 30s, 35 circulating, and finally 72℃ extending reaction 10 min, 4℃ conservation.

3.2.3 Cloning and sequences analyzing of chloroplast trnH-psbA gene
After purified the above-mentioned PCR product, the product connected with the carrier pMD19-T, and then connected to the cells of the feeling state top 10. Selected white spots to culture, then PCR detected bacterial colony, eventually sent purified product to Beijing Liuhe Huada gene polytron technologies Inc. for sequencing analysis.

3.2.4 Sequence analysis
The chloroplast trnH-psbA gene sequences of tested 67 samples were analyzed in this research. After removing the primer sequence in the sequencing results, Clustalx software was used for sorting, some loci should be revised manually, DNAMAN software was applied to analyze homologous analysis and PhyliP3.68 software was used for phylogenetic analysis. The phylogenetic tree were builded by parsimony approach. The calculation of the support rate by bootstrap for each branch was set to 1000 times repeated sampling.

Authors’ contributions
CJT did the data analysis, discussion of the results and drafted the manuscript. YXZ performed the experiments of the research; ZKZ conceived the project, guided the design of the experiments, revised the manuscript and is responsible for the project; JYH did the collection and the preparation of part of the experimental materials. All authors read and approved the final version of manuscript.

CJT and YXZ are the experimental designer and executive of this research; CJT and ZKZ did the data analysis and the draft; JYH involved in the experiment design and the test results analysis; ZKZ conceived the project, guided the design of experiments, analyzed the data, drafted and revised the manuscript. All authors read and approved the final version of manuscript.

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