Phylogenetic Analysis of Impatiens in the Eastern Yunnan, China

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Abstract

Impatiens L. is a genus of complex taxonomy that belongs to the family Balsaminaceae (Ericales) and contains approximately 1000 species. In this study, ISSR, *trnL-trnnF*, *atpB-rbcL* and *DEF* four molecular markers were used to analyze the genetic diversity and phylogenetic relationships among 32 species of Impatiens from eastern Yunnan. The results showed that the genetic similarity coefficient (GS) of these Impatiens resources ranged from 0.540 to 0.990, it indicated that the genetic diversity was relatively rich. However the genetic distance was relatively vague, and the evolutionary relationship was not obvious, which the phylogenetic trees were constructed by using the chloroplast genes *atpB-rbcL* and *trnL-trnF*. The genetic distance of the phylogenetic tree constructed by the *DEF* gene was relatively clear, the evolutionary relationship was obvious, and the result was consistent with ISSR. This indicated that *DEF* gene was greatly affected by plant habitat, and had abundant variation and a fast evolution rate, while *atpB-rbcL* and *trnL-trnF* genes had a slower evolution rate. And it was discovered that the flower morphological traits of the genus Impatiens can be employed as significant categorization markers. In particular, the wing shape can be used as an important flower morphological feature for the classification of the genus Impatiens in eastern Yunnan; and the results of using ISSR and *DEF* gene markers and flower morphological characteristics were consistent with the results of the interspecies division of Impatiens in this region. This study provided new basic data and a scientific basis for the protection, sustainable development, and utilization of Impatiens plant resources and genetic breeding in this region.

1 Introduction

Impatiens are mostly annual or perennial herbal ornamental flowers with vivid colors and peculiar flower shapes. There were more than 1,000 species of Impatiens in the world (Grey-Wilson 1980; Janssens et al. 2009), most of which were distributed in continental tropical, subtropical, and temperate regions. Its main distribution areas were tropical Africa, with about 109 species; Madagascar, with about 120 species; southern India and Sri Lanka, with about 150 species; Eastern Himalayas, with about 120 species; and Southeast Asia (Myanmar, Thailand, Southwest China, Indochina Peninsula and the Malaysian Islands), with about 300 species (Grey-Wilson 1980; Eberhard et al. 2015; Song et al. 2003). In China, the regional distribution of Impatiens was very obvious, and there were more than 270 species of Impatiens (Yu 2012), which were mainly distributed in the southwest and northwest. In particular, the southwestern provinces (Yunnan, Guangxi, Sichuan) were rich in species, and there were about 117 species in Yunnan at most (Li et al. 2013a). Among them, Impatiens species in eastern Yunnan account for 60% of the species, which was the region with the most concentrated distribution of Impatiens in Yunnan.

Linnaeus (1753) first described the genus Impatiens in Flora. Hooker et al. (1859) proposed a taxonomic overview of the genus Impatiens. Hooker (1973) then classified Impatiens according to fruit shape, phyllotaxy, inflorescence, sepals, and other characteristics. And others classified Impatiens into different groups and subgroups according to their morphological differences in seedlings (Shimizu 1982, 1985). The flower morphological structure (standard petal, wing petal, lip petal, sepal, etc.) of the genus Impatiens was an important taxonomic feature. Akiyama et al. (1991) divided the lip petal into three

types (navicular, funnel-shaped and cystic), which played an important role in classification. Akiyama et al. (2000) classified the inflorescences of 39 Impatiens species in the Himalayas into 9 types based on differences in bracts, inflorescences, and pedicels. Chen (2001) adopted the viewpoint of Hooker (1973) and identified 220 species of Impatiens. Yu et al. (2007) discovered that the various characteristics of pollen can be used to identify new species. Shimizu et al. (1982, 1984, 1985, 1996) dissected the pistils of 6 species of Impatiens in India, and pointed out that the Impatiens with connate and detached-wings had different carpels and ventricles. Chen (2007) investigated the pistils of the genus Impatiens and demonstrated that the number of carpels, chambers in the ovary, and ovules can also be used for Impatiens classification and identification.

The number of chromosomes in the genus Impatiens varies greatly (Akiyama et al. 1992; Sugawara et al. 1994, 1997; Shimizu 2017; Liang et al. 2022). Fang (2011) analyzed the chromosomes of 7 Impatiens species in Jiangxi and discovered that the more symmetrical the karyotype, the lower the evolutionary degree. Jeelani et al. (2010) discovered that chromosome number variation played an important role in Impatiens evolution, and that morphological differences between different species of the genus were closely related to the number of chromosomes. The chloroplast genome (CpDNA) and nuclear genome were currently widely used in plant genetic diversity, phylogenetic relationships, and lineage geography. Luo et al. (2021a, b) sequenced the chloroplast genome of some Impatiens and identified some long repeat sequences and simple repeats, which provided valuable and important genomes for Impatiens species identification and phylogenetic evolution. Our research aimed to systematically investigate Impatiens in eastern Yunnan, and provide basic data for the protection of Impatiens resources and species diversity in this area. ISSR, *trnL-trnnF*, *atpB-rbcL*, and *DEF* sequences were used to explore genetic diversity and species identification. We attempted to identify the most important morphological classification markers and the most suitable molecular markers of Impatiens in eastern Yunnan by combining traditional classification and identification methods with molecular markers.

Genetic maps were an important tool for genetics and genomics research in Impatiens plants. Geuten et al. (2004) proposed that the Impatiens family was monophyletic based on the CpDNA labeling method. Yuan et al. (2004) also proved that Balsamaceae was a monophyletic group based on the *ITS* sequence, and speculated that the genus Impatiens was also a monophyletic group. Janssens et al. (2006b) used the *atpB-rbcL* gene sequence to confirm that the genus Impatiens and Hydrocera were sister groups. Yu et al. (2015) conducted phylogenetic analysis on 150 Impatiens species and three outgroups using *ITS* and chloroplast *atpB-rbcL* and *trnL-trnF* sequences, and the results revealed that Impatiens evolved into two major groups. Provan et al. (2007) studied the *I. glandulifera* population using ISSR and discovered that its genetic diversity was relatively rich. Li et al. (2013b) used ISSR molecular marker technology to study the population of *I. macrovexilla* in Guilin and found that its genetic diversity was relatively low. Zhong et al. (2014) used the ISSR method to study the genetic diversity of the Hainan Impatiens population at different altitudes, and found that altitude was a significant factor affecting the gene flow of the population. Abbasi et al. (2022) used ISSR to analyze the genetic diversity of *Juglans regia* L. (Persian walnut), demonstrated the significant genetic variety of the Azarshahr population and provided crucial data for breeding and resource preservation. The research on Impatiens' genetic diversity also made

extensive use of additional molecular markers. Jason et al. (2003) used AFLP molecular markers to analyze the genetic diversity of 41 New Guinea Impatiens cultivars. Xie (2011) successfully identified and analyzed Impatiens mutants by ISSR and RAPD methods. Mcabee et al. (2005) used the *INO* ovule development gene to conduct related research on the genus Impatiens, and believed that the integument of the genus was characterized by only one layer as a relatively primitive species. *APETALA3 (AP3)/DEFICIENS (DEF*) was a MADS-box transcription factor that can be used to determine the identity of petal and stamen flower organs, and this gene profile has been widely used in plant genetic diversity, phylogenetic relationships, and lineage geography field. Janssens et al. (2006a) found that the 4th and 5th introns of the K domain of the *AP3/DEF* gene can be used as an important basis for the classification of Impatiens. Xia (2020) used *DEF* sequence markers and found that the sub. *Clavicarpa* was a monophyletic group.

2 Materials And Methods

2.1 Plant materials

In this study, a total of 32 Impatiens species were collected from eastern Yunnan (Zhaotong, Qujing, Wenshan, Honghe and Kunming), and their origin location, altitude, plant type height, and environment were recorded. (The results are shown in Table 1) And their total DNA was extracted and placed at -80℃ for long-term storage. The DNA extraction method was followed per the instructions of the Biotech DNA extraction kit, and the quality of DNA was detected by 1.0% agarose gel electrophoresis.

2.2 The establishment of ISSR-PCR amplification system and analysis of genetic diversity

The ISSR reaction primers used a total of 23 sequences provided by Columbia University (UBC), and the relevant information was shown in Table 2. The PCR reactions (20 μL) contained 1.5 uL template DNA, 2.0 uL of 10 × Buffer (Mg^{2+} plus), 1.6 uL dNTP Mixture (2.5 mM per), 0.1 uL TaKaRa Taq (5 U/uL), 1.0 uL of each primer pair and 13.8 uL dd H_2O. The thermal cycling parameter was an initial cycle of 2 min at 94 ℃, followed by 40 cycles of 98 ℃ for 10 sec, annealing temperature (Ta) for 30 sec, 72 ℃ for 1 min plus a final extension of 72 ℃ for 10 min. Data were statistically analyzed using NTSYS-PC V2.1. The presence or absence of the relative migration position of the amplified band on the agarose gel map was marked as "1" or "0", respectively. According to the GD value, the genetic similarity cluster analysis was carried out on 32 species of Impatiens in Yunnan province by the unweighted group average method (UPGMA), and the genetic relationship dendrogram was drawn. According to Nei's method, the genetic similarity coefficient (GS) between materials was calculated, and the calculation formula was:

$$GS = \frac{2N_{ij}}{N_i + N_j}$$

(Note: Nij is the number of amplified bands shared by materials i and j, Ni is the number of amplified bands of material i, and Nj is material j the number of amplified bands, the genetic distance GD = 1-GS.)

2.3 Analysis of the genetic relationship based on *atpB-rbcL*, *trnL-F* and *DEF* gene sequence

The Impatiens' *atpB-rbcL* gene sequence primers were *atpB* (5'-ACATCTAGTACCGGACCAATGA-3') and *rbcL*

(5'-AACACCAGCTTTGAATCCAA-3'). The Impatiens' tr*nL-F* gene sequence primers were *trnL* (5'-CGAAATCGGT

AGACGCTACG-3') and *trnF* (5'-ATTTGAACTGGTGACAGAG-3'). The Impatiens' *DEF* gene sequence primers were *DEF*-F (5'-GCAGATACACAGAAACCTACTTCAG-3') and *DEF*-R (5'-TTATAGTTAAAGCAAAGCATAGGT

TGTGAG-3'). The PCR reactions (20 μL) contained 1.5 uL template DNA, 2.0 uL of 10 × Buffer (Mg^{2+} plus), 1.6 uL dNTP Mixture (2.5 mM per), 0.1 uL TaKaRa Taq (5 U/uL), 0.5 uL of each primer pair and 13.8 uL dd H_2O. The thermal cycling parameter was an initial cycle of 2 min at 94 ℃, followed by 30 cycles of 94 ℃ for 1min, annealing temperature (Ta) for 30 sec, 72 ℃ for 1 min plus a final extension of 72 ℃ for 10 min. The Tas of *atpB-rbcL*, *trnL-F*, and *DEF* gene sequences were 51 ℃, 55 ℃, and 52.5 ℃. The PCR products were sequenced after detection by 1.5% agarose gel electrophoresis. In MEGA 10.0, the NJ method was used to construct a phylogenetic tree based on the gene sequence for the genus of Impatiens in eastern Yunnan. Using the Bootstrap method to test the confidence of each branch in the tree, and the number of bootstraps was 1000.

3 Results

3.1 Species and geographical distribution of Impatiens plant resources in Eastern Yunnan

East Yunnan was located in the southwest of China, and had a relative altitude difference of about 4000m, with the highest altitude of 4040m and the lowest altitude of 107m. This region was influenced by both high and low latitudes and had a three-dimensional climate, which provided unique conditions for the growth and development of Impatiens. The 32 species of Impatiens collected this time were mainly concentrated in the high-humidity areas with an altitude of 1600 ~ 3000m, of which 2 species (*I. dichroa* and *I. napoensis*) were distributed at an altitude of 1300 ~ 1600m, accounting for about 6.25%; of which 9 species (*I. rubrostriata*, *I. mengtzeana*, *I. linearisepala*, etc.) were distributed at an altitude of 1600 ~ 2000m, accounting for about 28.125%; of which 20 species (*I. racemosa*, *I. pinfanensis*, *I. desmantha*, etc.) distributed at an altitude of 2000 ~ 3000m, accounting for about 62.5%; of which only *I. rubrostriata* was distributed above 3000m, accounting for about 3.125%.

From the perspective of the lateral distribution of species and the number of individuals, Kunming, Zhaotong and

Wenshan were the main distribution centers of Impatiens. Among them, 10 species of Impatiens were gathered in Zhaotong and Qujing regions, 10 species were collected in Wenshan and Honghe areas, and 12 species collected in Kunming and surrounding areas. The 32 Impatiens resources collected this time accounted for 70% of the total in eastern Yunnan. This study also found that *I. siculifer*, *I. racemosa*, and *I. radiata*, which are widely distributed laterally, had certain differences in their growth traits in the same ecological type. (The results are shown in Table 1)

Tab 1

Distribution information of 32 species/varieties of Impatiens from eastern Yunnan

NO.	Species	Origin	Height/cm	altitude/m	Habitat
1	*I. radiata*	Zhaotong	40 ~ 60	2681 ~ 2812	Roadside hillside wet slope
2	Unidentified Species 1	Zhaotong	45 ~ 60	2632 ~ 2828	Roadside hillside wet slope
3	*I. pinfanensis*	Zhaotong	20 ~ 40	2750 ~ 2916	Roadside hillside wet slope
4	*I. arctosepala*	Zhaotong	20 ~ 30	2589 ~ 2746	Beside the mountain stream
5	*I. cyathiflora*	Zhaotong	30 ~ 60	2643 ~ 2720	Beside the mountain stream
6	*I. desmantha*	Zhaotong	30 ~ 60	2437 ~ 2658	Beside the creek
7	*I. dicentra*	Zhaotong	60 ~ 90	2398 ~ 2661	Beside the creek
8	*I. oxyuanthera*	Zhaotong	20 ~ 40	2544 ~ 2663	Beside the creek
9	*I. alpicola*	Daguan County	20 ~ 30	1736 ~ 1942	Roadside creek
10	*I. dichroa*	Daguan County	30 ~ 40	1342 ~ 1472	Roadside hillside wet slope
11	*I. siculifer. var.*	Pingbian County	50 ~ 60	1454 ~ 1602	Roadside hillside wet slope
12	*I. siculifer*	Pingbian County	50 ~ 60	1355 ~ 1602	Roadside hillside wet slope
13	*I. linearisepala*	Pingbian County	30 ~ 40	1521 ~ 1602	Roadside hillside wet slope
14	*I. napoensis. var.*	Pingbian County	60 ~ 90	1430 ~ 1615	Roadside hillside wet slope
15	Unidentified Species 2	Daweishan Nature Reserve	50 ~ 70	1753 ~ 1978	Roadside hillside wet slope
16	*I. racemosa*	Daweishan Nature Reserve	20 ~ 60	1833 ~ 2026	Roadside hillside wet slope
17	*I. napoensis*	Daweishan Nature Reserve	60 ~ 90	1205 ~ 1380	Roadside creek
18	*I. duclouxii*	Pingbian County	20 ~ 40	1468 ~ 1683	Roadside creek
19	*I. mengtzeana*	Pingbian County	40 ~ 60	1678 ~ 1857	Roadside dry river
20	Unidentified Species 3	Xiaojie town	50 ~ 70	1654 ~ 1841	Roadside hillside wet slope
21	*I. longialata*	Xundian County	30 ~ 70	2038 ~ 2279	In the grass by the creek
22	*I. polyceras*	Xundian County	20 ~ 75	1844 ~ 2279	In the grass by the creek
23	*I. apsotis*	Xundian County	10 ~ 30	1974 ~ 2280	In the grass by the creek
24	*I. blepharosepala*	Xundian County	30 ~ 60	1899 ~ 2280	In the grass by the creek
25	*I. bodinieri*	Xundian County	30 ~ 50	2031 ~ 2345	In the grass by the creek
26	*I. aquatilis*	Luquan County	30 ~ 50	2135 ~ 2483	By the ditch under the hillside by the roadside
27	*I. corchorifolia*	Luquan County	30 ~ 50	2032 ~ 2292	In the grass on the hillside by the road
28	Unidentified Species 4	Sedan chair snow mountain	40 ~ 70	2654 ~ 2868	Mountain stream grass
29	*I. vittata*	Sedan chair snow mountain	30 ~ 60	2596 ~ 2857	Mountain stream grass
30	*I. rubrostriata*	Luquan County	30 ~ 80	3166 ~ 3437	Roadside hillside grass
31	Unidentified Species 5	Songming County	30 ~ 60	1967 ~ 2118	Roadside sparse broad-leaved forest
32	*I. uliginosa*	Kunming	60 ~ 80	2034 ~ 2151	Beside the creek

3.2 Analysis of flower morphological characteristics of Impatiens in Eastern Yunnan

The genus Impatiens in eastern Yunnan had peculiar flower shapes and a wide range of vibrant colors. Among them, 15 species of yellow series account for 46.88%, 8 species of red series account for 25.00%, 7 species of white series account for 21.88%, and 2 species of purple series account for 6.25% of the total. In this investigation, it was found that the flower organs of the genus Impatiens had

heterochromatic spots or stripes. The anatomical observation of flower morphology found that there were 2 or 4 sepals in the genus Impatiens, and most of the Impatiens with 2 sepals, *I. linearisepala* and *I. corchorifolia* has 4 sepals. The shape of standard shape of the genus Impatiens in eastern Yunnan was typically circular or nearly circular. Its wing lobes were all two-lobed, though some were three-lobed or more, and the base lobes were oval, nearly oval, nearly round, round, etc; the upper lobes were ax-shaped, broad ax-shaped, narrow ax-shaped, nearly elliptical, and half-moon-shaped; the shape of the lip shape was sac-shaped, funnel-shaped, narrowly funnel-shaped, boat-shaped, etc.

3.3 Polymorphism of ISSR markers

ISSR polymorphism analysis of Impatiens in eastern Yunnan revealed that 23 ISSR primers identified a total of 235 high allelic richness, 223 polymorphic bands, and a relatively high polymorphism ratio of 0.9490. Sites with a high polymorphism (66.7%). Primer UBC825 had the least polymorphic site, amplified 6 polymorphic bands, and the polymorphism ratio of 14 primers reached 100%, accounting for 60.9% of the total, with primers UBC848 and UBC861 amplifying up to 15 bands. And primers UBC848 and UBC811 could well distinguish 32 species of Impatiens in eastern Yunnan(The results are shown in Fig. 1). Therefore, the polymorphic loci in the genus Impatiens revealed the polymorphism difference of the genus,it indicated that the species richness of the genus Impatiens was higher in eastern Yunnan. (The results are shown in Table 2)

Tab 2

ISSR-PCR primers and their amplification results

NO.	Primer sequences(5'to 3')	Tm(°C)	mplified sites	polymorphic sites	polymorphic site ratio
UBC825	ACACACACACACACACT	52.2	9	6	66.7%
UBC835	GAGAGAGAGAGAGAGAG	56.2	8	6	75.0%
UBC871	TATTATTATTATTATTAT	34.5	8	7	87.5%
UBC810	GAGAGAGAGAGAGAGAT	52.2	8	7	87.5%
UBC880	GGAGAGGAGAGGAGA	53.6	9	8	88.9%
UBC868	GAAGAAGAAGAAGAAGAA	48.2	10	9	90.0%
UBC834	AGAGAGAGAGAGAGAGYT	53.9	11	10	90.9%
UBC864	ATGATGATGATGATGATG	48.2	11	10	90.9%
FXH214	GAGGAGGAGGAGGAGGAG	61.9	11	10	90.9%
UBC807	AGAGAGAGAGAGAGAGT	52.2	11	11	100%
UBC811	CACACACACACACACAA	54.6	6	6	100%
UBC817	CACACACACACACACAA	52.2	13	13	100%
UBC841	GAGAGAGAGAGAGAGAYG	56.2	13	13	100%
UBC842	GAGAGAGAGAGAGAGAYG	56.2	13	13	100%
UBC848	CACACACACACACACARG	56.2	15	15	100%
UBC861	ACCACCACCACCACCACC	61.9	15	15	100%
UBC862	AGCAGCAGCAGCAGCAGC	61.9	9	9	100%
UBC867	GGCGGCGGCGGCGGCGGC	75.5	10	10	100%
UBC888	BDBCACACACACACACA	53.8	9	9	100%
UBC889	DBDACACACACACACAC	53.0	9	9	100%
UBC890	VHVGTGTGTGTGTGTGT	53.0	9	9	100%
UBC891	HVHTGTGTGTGTGTG	52.6	8	8	100%
FXH215	AGGAAGGAAGGAAGGAAGAA	57.8	10	10	100%
Total	–	–	235	223	94.9%
Average	–	–	10.22	9.70	94.9%

Cluster analysis was performed based on the genetic similarity coefficient of Impatiens in eastern Yunnan (Fig. 2), with GS values ranging from 0.540 to 0.990, it indicated significant genetic differences. The dendrogram was divided into three groups, with Group I divided into 4 subclades, SCI, SCII, SCIII, and SCIV. For SCI, accessions such as *I. apsotis*, *I. longialata*, and *I. polyceras* were all collected in Xundian County; *I. siculifer* var., unidentified species 2, *I. racemosa*, *I. duclouxii*, *I. napoensis* and *I. napoensis* var. were all collected in Pingbian County. *I. duclouxii*, *I. napoensis* and *I. napoensis* var. were tightly clustered together, indicating that the three Impatiens species were closely related, and its flowers were larger, with

round standard petals, short-handled, two-lobed petals, wide funnel-shaped blades, and very similar flower shapes. However, *I. Siculifer* and *I. siculifer* var. were not closely clustered, indicating that the variation of ISSR polymorphisms was larger than that of *I. napoensis* and *I. napoensis* var.. In SCII, *I. linearisepala* was divided into a single group, and it has 4 sepals, indicating that the number of sepals was also an important taxonomic indicator of the genus in eastern Yunnan. Similarly, *I. oxyuanthera* in SCIII was grouped separately. In SCIV, unidentified species 1, *I. dicentra*, *I. pinfanensis*, *I. arctosepala*, and *I. desmantha* were all collected in Zhaotong, indicating the genetic relationship among the genus Impatiens in the same area closer. Group II consisted of the unidentified species 5 and *I. uliginosa*, indicating that they were closely related, which provided a certain basis for the identification of the unidentified species 5. Group III contained *I. mengtzeana* and *I. aquatilis*, and they were all pink flowers of the balsam genus. (The results are shown in Fig. 2)

3.4 *atpB-rbcL* sequence polymorphism marker

A dendrogram analysis using *atpB-rbcL* markers identified five major groups (Fig. 3), also highlighting the significant territorial characteristics of of Impatiens in eastern Yunnan. For group I, three sub-clades of SCI, SCII, and SCIII were detected, and most of them were collected in Pingbian County and Xundian County. In SCI, *I. polyceras*, *I. rubrostriata*, unidentified species 2, *I. siculifer*, *I. siculifer* var., *I. dichroa*, *I. aquatilis*, *I. mengtzeana*, and unidentified species 3 were all clustered together, indicating that these species of Impatiens plants were closely related.

Among them, *I. siculifer* and *I. siculifer* var. were closely clustered, which differed from the ISSR clustered results; unidentified species 3 was closely clustered with *I. siculifer* and *I. mengtzeana* respectively in the two markers, which made the identification of its species more difficult. However, *I. aquatilis* and *I. mengtzeana* were closely clustered in the evolutionary tree of ISSR and *atpB-rbcL* sequence markers. In SCII, *I. Cyathiflora* was grouped separately, indicating that it was far from relatives of the genus *Impatiens* in SCI. In SCIII, it consisted of *I. racemosa*, *I. longialata*, *I. arctosepala*, *I. radiata*, *I. apsotis*, and unidentified species 4 were clustered together; among them, *I. racemosa* and *I. longialata* were closely clustered, indicating that the *atpB-rbcL* clustered was more closely related than ISSR clustered; the remaining three were consistent with the ISSR clustered results and were all closely clustered. Group II was divided into 2 subclades, SCI and SCII. In SCI, *I. uliginosa* and unidentified species 5 were grouped, which was consistent with the ISSR results, indicating that they were closely related. Therefore, it was speculated that the unidentified species 5 was very likely to be a variant of *I. uliginosa*. In SCII, *I. napoensis* var., *I. napoensis*, *I. duclouxii*, *I. dicentra*, *I. corchorifolia*, unidentified species 1, *I. blepharosepala*, *I. pinfanensis*, *I. vittata*, *I. oxyuanthera*, *I. desmantha*, *I. alpicola*, and *I.bodinieri* were clustered together; and *I. napoensis* var., *I. napoensis*, *I. duclouxii* were closely clustered, which was consistent with the ISSR results, indicating that their kinship was closer, and the clustered results were more reliable. Group III, *I. linearisepala* was clustered individually. In conclusion, these two molecular markers have high reliability for the genetic diversity analysis of Impatiens in eastern Yunnan.

3.5 *trnL-trnF* sequence polymorphism marker

The dendrogram was mainly divided into 3 groups. For group I was divided into 2 subgroups, SCI and SCII. In SCI, *I. cyathiflora*, *I. aquatilis*, unidentified species 3, *I. mengtzeana*, *I. dichroa*, *I. siculifer*, *I.siculifer* var., *I. polyceras*, unidentified species 2, and *I. rubrostriata* were clustered together; in SCII, *I. radiata*, unidentified species 4, *I. apsotis*, *I. arctosepala*, *I. racemosa* and *I. longialata* were clustered together, which the clustered results were the same as that of *atpB-rbcL* sequence clustered. And *I. polyceras*, unidentified species 2, *I. rubrostriata* were closely clustered together. While *I. cyathiflora* was not clustered individually among the *trnL-trnF* sequence polymorphism markers. In group II, *I. bodinieri*, *I. napoensis*, *I. napoensis* var. and *I. duclouxii*, unidentified species 5, *I. uliginosa*, unidentified species 1, *I. corchorifolia*, *I. dicentra*, *I. blepharosepala*, *I. alpicola*, *I. desmantha*, *I. vittata*, *I. pinfanensis*, and *I. oxyuanthera* were gathered together. The 15 species of Impatiens and the 15 species of Impatiens marked by *atpB-rbcL* sequence were homogenized into a group, it indicated that the clustering results of these two markers were consistent, and the chloroplast genome sequences (*atpB-rbcL*, *trnL-trnF*) could be used for the evolutionary analysis of Impatiens in eastern Yunnan. And in the *atpB-rbcL* sequence marker, *I. alpicola* and *I. bodinieri* were closely clustered together, indicating that their kinship was closer and the evolutionary relationship was obvious; but in the *trnL-trnF* sequence marker, the evolutionary relationship of *I. bodinieri*, *I. dicentra*, *I. blepharosepala*, etc. was not obvious. It indicated that the *atpB-rbcL* sequence marker was more effective for the genetic diversity analysis of the genus Impatiens in eastern Yunnan. In group III, *I. linearisepala* was grouped individually. Among group II, *I. napoensis*, *I. napoensis* var. and *I. duclouxii* were closely clustered in the three markers, and the unidentified species 5 and *I. uliginosa* were also closely clustered in the three markers; it showed that ISSR and the chloroplast genome sequences had certain applicability to the evolution analysis of Impatiens in eastern Yunnan, but the evolutionary relationship of chloroplast genome sequence markers was not obvious, while the genetic distance of ISSR was relatively clear, the evolutionary relationship was more obvious. (The results are shown in Fig. 4.)

3.6 *DEF* sequence molecular marker

The dendrogram was mainly divided into 4 groups. Group I was divided into 4 subgroups, SCI, SCII, SCIII, and SCIV. In SCI, *I. oxyuanthera*, *I. alpicola*, *I. pinfanensis*, *I. vittata* were clustered together; and *I. desmantha*, *I. bodinieri* were closely clustered, which was consistent with the results of *atpB-rbcL* and ISSR sequence clustered, indicating that they were closely related; and *I. blepharosepala*, *I. siculifer*, *I. arctosepala*, unidentified species 3 were tightly clustered together, which was consistent with the ISSR sequence clustered results. In SCII, *I. dicentra* was individually clustered into a subgroup. In SCIII, *I. corchorifolia* and unidentified species 3 were tightly clustered together. In SCIV, *I. uliginosa* and unidentified species 5 were tightly clustered, and *I. duclouxii*, *I. napoensis*, and *I. napoensis* var. were closely clustered, which was consistent with the other three marker results, further illustrating the reliability of the clustered results. The *DEF* sequence marker indicated that the five species were closely related to each other, and it were consistent with the *trnL-trnF* sequence results. However, in the *atpB-rbcL* sequence and ISSR sequence markers, *I. uliginosa*, unidentified species 5, *I. duclouxii*, *I. napoensis*, and *I. napoensis* var. were clustered into different groups, reflecting the clustering differences between the

different markers. In group II, *I. mengtzeana* and *I. aquatilis* were grouped separately in a group, and the results were consistent with the ISSR. Using *atpB-rbcL* sequence, *I. mengtzeana*, *I. aquatilis*, and unidentified species 3 were closely clustered together; and their evolutionary relationship of them in *trnL-trnF* marker was not obvious. These results indicated that the nuclear gene sequence markers were more obvious in the evolutionary relationship of Impatiens in eastern Yunnan. For group III was divided into 3 subgroups, SCI, SCII, and SCIII. In SCI, *I. cyathiflora* clustered separately into a subgroup. In SCII, *I. radiata*, unidentified species 4, *I. longialata*, *I.apsotis*, and *I. racemosa* were grouped. However, in the chloroplast genome sequence markers, *I. arctosepala* was also closely clustered with these five species, which was different from *DEF* and ISSR sequence markers. Although these five species of Impatiens were clustered into a group in ISSR sequence markers, their clustering results were distant and their genetic relationship was unclear. In SCIII, *I. siculifer* var., undetermined species 2, *I. polyceras*, and *I. rubrostriata* were clustered together; but the clusters of *I. siculifer* and *I. siculifer* var. were far apart, indicating greater changes in nuclear genes. In group SCIV, *I. linearisepala* singly gathered into a group. These results indicated that the *DEF* sequence marker can better explain the relationship among Impatiens in this area. (The results are shown in Fig. 5.)

4 Discussion

Impatiens species in China were mainly distributed in the southwest region, especially in the east and southeast of the Qinghai-Tibet Plateau, where they were mostly distributed at an altitude of 1000 ~ 4000m, such as in Hengduan Mountains (Yu. 2012). However, the classification of Impatiens has always been a difficult problem. The morphological variation of Impatiens was very complex, and the variation of chromosome cardinality was quite different. Jeelani et al. (2010) showed that changes in chromosome number played an important role in the evolution of Impatiens, and that morphological differences between species were closely related to the number of chromosomes. And Fujihashi et al.(2002) found that the number of chromosomes and inflorescence morphology showed a certain evolutionary trend. Song et al. (2003) found that the chromosome number of Chinese Impatiens was 2n = 12, 14, 16, 18, 20, 40, 54, etc. While, the three common chromosome bases of Impatiens in the Himalayan mountains were x = 7, 9, and 10 (Akiyama 1992; Akiyama 2000; Ikeda 2005). These results indicated that the common chromosome cardinality was correlated with its geographical distribution, indicating the diversity of chromosome cardinality in Impatiens. And the distribution of Impatiens had a significant region, and its narrow and unique phenomenon was very obvious, and with specialized pollinators, interspecific hybridization was very difficult. However, similar habitat conditions lead Impatiens species to evolve in similar morphological directions, which made it difficult to classify Impatiens species. Therefore, when studied the classification of Impatiens plants, using morphological characteristics such as seedling morphology, labellus, bracts, inflorescences, and pedicel as the basis for classification was completely inadequate (Akiyama 1991; Akiyama et al. 2000; Hooker 1973).

The survey plot in this study was Yunnan Province, which was rich in Impatiens plant resources with about 110 species (Li et al. 2013a). A total of 32 Impatiens species were collected in eastern Yunnan, mainly distributed in the high humidity area with an altitude of 1600 ~ 3000m. All 32 Impatiens species

were endemic to southwest China, and 9 species were endemic to Yunnan Province. The genetic similarity coefficient (GS) of Impatiens in eastern Yunnan ranged from 0.540 to 0.990. Compared to *I. glandulifera* (Provan et al. 2007), *I. macrovexilla* (Li et al. 2013b), and Impatiens (Yu et al. 2021) discovered that the genetic similarity coefficient of Impatiens resources in eastern Yunnan was higher, and species resources were more abundant in this region. Cluster analysis of ISS, *atpB-rbcL*, *trnL-trnF*, and *DEF* sequence markers as well as flower morphological characteristics (wing lobe shape, flag lobe shape, labial lobe shape, flower size, etc.) revealed a significant correlation between them on the interspecific division of Impatiens in eastern Yunnan. In contrast, Fujihashi et al. (2002) stated that there was no obvious correlation between the lower sepal and the evolutionary tree. Impatiens distribution in eastern Yunnan was remarkably regional. The results of four molecular markers showed that the species with similar biological characteristics and habitat conditions clustered together, which reflected the genetic similarity between different evolutionary groups. In addition, related studies have shown that different bracts, pedicels (Akiyama et al. 2000), pistils (Shimizu et al. 1982, 1984, 1985, 1996), and inflorescence patterns. Gao (2012) also considered flower morphologyan as an important characteristic of the genus classification. The results confirmed that flower morphology was the key character for the division of species in this genus, especially the flap shape can be used as an important taxonomic character in the classification of Impatiens in eastern Yunnan.

In the dendrogram of genetic phylogenetic analysis based on *atpB-rbcL* and *trnL-F* genes, it was found that the same flower color, lip shape, and flower size could not made Impatiens in eastern Yunnan area cluster together, and some groups had limited support in the evolutionary tree, and the genetic distance was vague, and the evolutionary relationship was not obvious. Azizi et al. (2022) also stated that the CpDNA dendrogram was less useful for Tragopogon classification, and because the chloroplast sequences of Tragopogon species were very similar, it was not recommended to use CpDNA markers in the classification of this genus. However, Gao et al. (2012) showed that *ITS* and *atpB-rbcL* sequences had high reliability in the classification of *I. balsamina*, which was speculated to be related to species differences and regionalism. These results indicated that the chloroplast *atpB-rbcL* and *trnL-F* genes were not affected by plant habitat during the growth process of Impatiens in eastern Yunnan, and their gene evolution rate was slow and the degree of variation was general. They might not accurately reflect the phylogenetic relationship of Impatiens in eastern Yunnan. Based on ISSR and phylogenetic tree analysis of floral development regulation *DEF* gene, it was found that these two markers were not only consistent with the morphological characteristics of Impatiens plants in this area but also revealed some genetic relationships among different Impatiens species in this area. It also indicated that some floral features of this genus, such as wing shape, standard shape, and lip shape could be used as important taxonomic features. And the variability of the *DEF* gene fragment in Impatiens plants in eastern Yunnan was great, indicating that the *DEF* gene fragment was greatly affected by plant habitat, and its evolution rate was fast. Furthermore, the support rate in the phylogenetic tree was high, the genetic distance was clear, and the genetic relationship was obvious, which reflected the genetic relationship of these 32 species (or varieties) in eastern Yunnan, which was consistent with the research results of Janssens' results (Janssens. et al. 2006a).

A total of 2 varieties and 5 unconfirmed species were collected in this study. The cluster analysis showed that the *I. siculifer* var. and *I. siculifer* were closely clustered together, and the *I. napoensis* var. and *I. napoensis* were closely clustered together, which further verified their close relationship, and determined that they were respectively the *I. siculifer* var. and *I. napoensis* var.. The clustering results also showed that the undetermined species 1 was the most closely related to *I. corchorifolia*, and it was speculated that it might be a new species in Section Impatiens or a variety of *I. corchorifolia*. The undetermined species 2 was closely related to *I. polyceras* and *I. rubrostriata*, so it might be a new species in Section Scorpioid-cyma. In the CpDNA dendrogram, undetermined species 3 was closely related to *I. aquatilis*, *I. mengtzeana*, and *I. siculifer* var.; and in the cluster of flower morphology, it clustered with *I. mengtzeana* and *I. siculifer* var.; but in the nuclear gene dendrogram, it was more closely related to *I. siculifer* and *I. arctosepala.* Therefore, the category of undetermined species 3 could not be well identified. And undetermined species 4 was closely related to *I. radiata* and *I. apsotis.* All of them were white and small flowers, it was difficult to identify them. Undetermined species 5 was closely clustered with *I. uliginosa* and was the closest relative. It may be a variety or an evolutionary species of Section Racemus *I. uliginosa.*

5 Conclusion

In this study, we systematically collected 32 species of Impatiens in eastern Yunnan and found that they had remarkable regional characteristics. The results of ISSR polymorphism sequence markers showed a high genetic similarity coefficient and a high genetic diversity. It was found that *atpB-rbcL*, *trnL-trnF*, and *DEF* sequence markers and floral morphological characteristics were significantly correlated with an interspecific division of Impatiens in eastern Yunnan. And the characteristic of flap shape was more prominent in the classification of the Impatiens. Cluster analysis of four molecular markers showed that the genetic distance of the phylogenetic tree constructed by chloroplast genes *atpB-rbcL* and *trnL-trnF* was fuzzy and the evolutionary relationship was not obvious; while the phylogenetic tree constructed by *DEF* gene showed clear genetic distance and obvious evolutionary relationship, indicating that *DEF* gene sequence markers were extremely important in the identification and evolutionary analysis of Impatiens in eastern Yunnan. Despite the limitations of the materials used in this experiment, the results were consistent with the morphological characteristics and classification of the genus, and to some extent, the genetic relationship between the Impatiens was revealed. These findings indicated that ISSR and floral development regulation of the *DEF* gene had significant value and significance in exploring Impatiens' genetic relationships in this region. Simultaneously, how to improve Impatiens classification in eastern Yunnan and how to select more appropriate marker genes must be discussed and researched further.

Declarations

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Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by **Fan Li, Rui Zhao, Yang Li, Xingyi Li, Chunmei Wei, Xiaoli Zhang, Haihao He, Suping Qu, Meijuan Huang, Haiquan Huang**. The first draft of the manuscript was written by **Fan Li** and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability Statements

1.The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

2.All data generated or analyzed during this study are included in this published article (and its supplementary information files).

3.The datasets generated during and/or analyzed during the current study are not publicly available due to [REASON(S) WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.

4.Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Figures

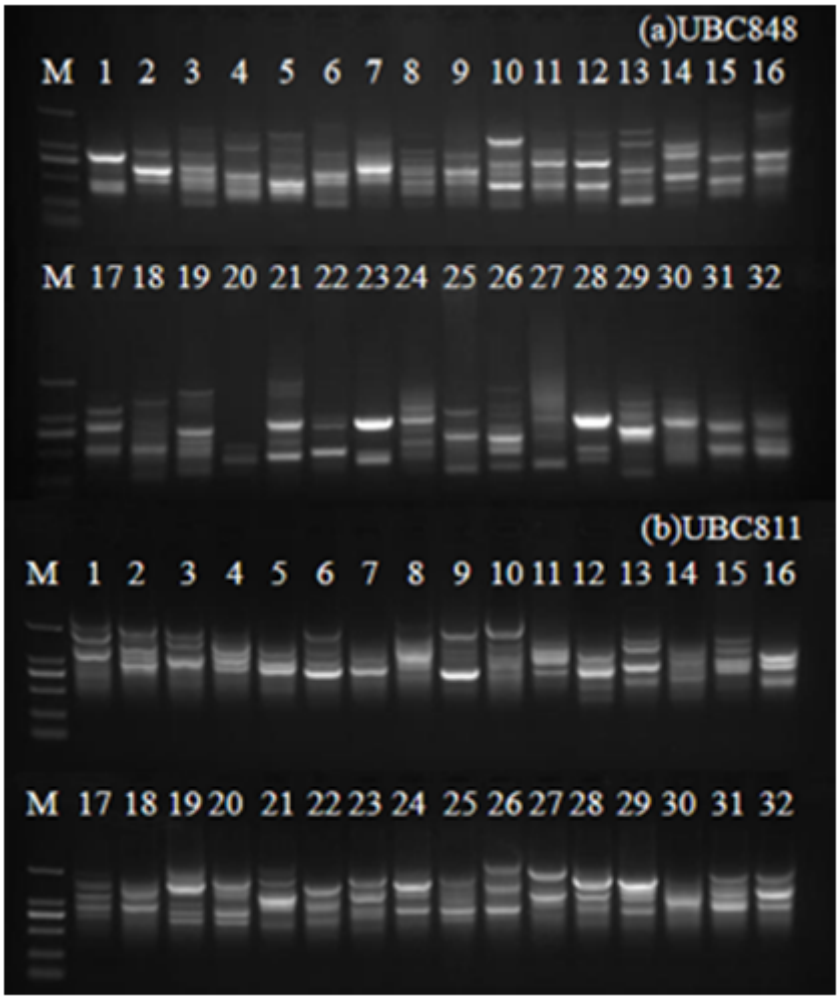

Figure 1

Amplification profile of 32 Impatiens samples using UBC848(a) and UBC811(b) primers

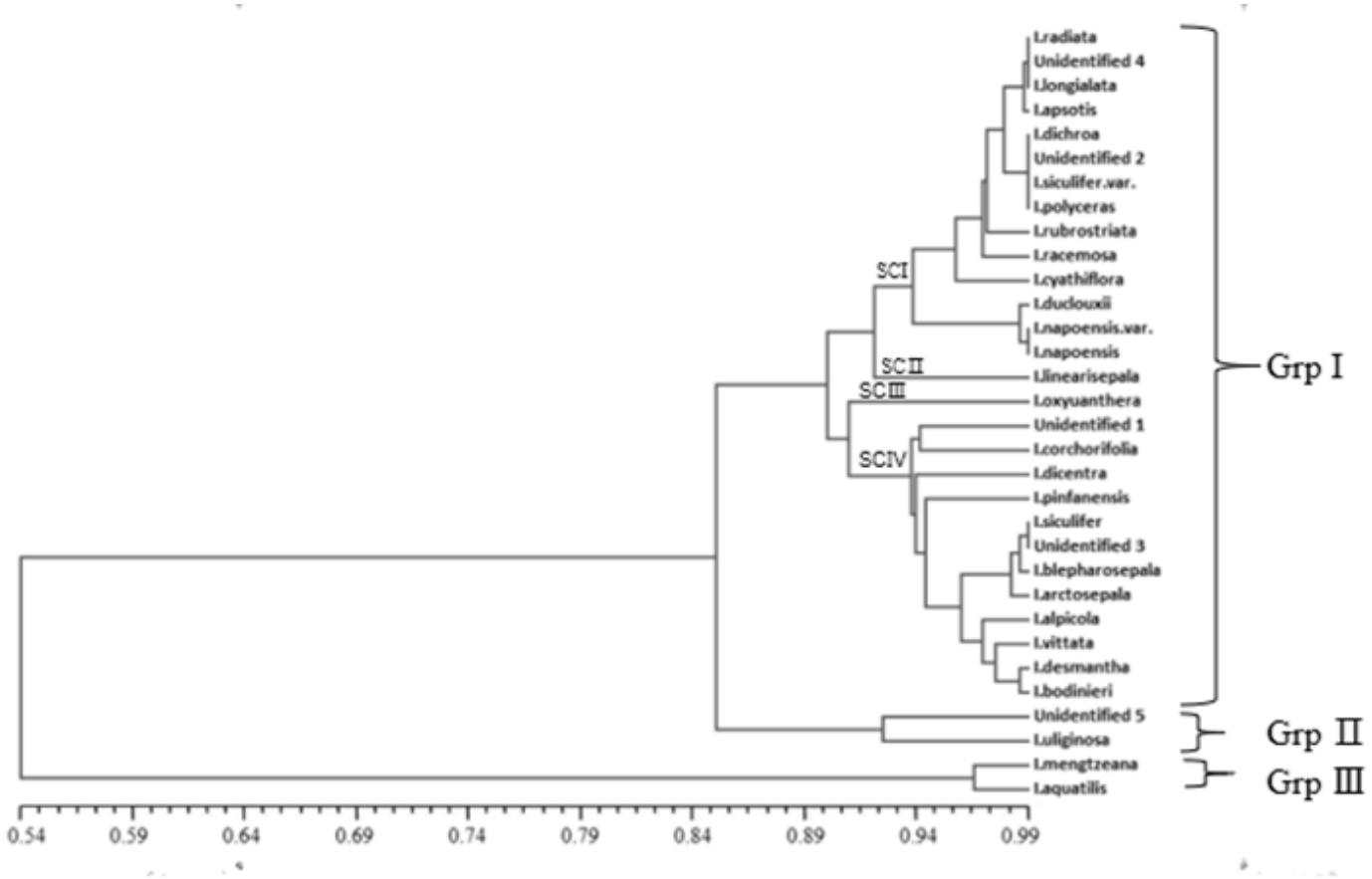

Figure 2

UPGMA-derived dendrogram of 32 *Impatiens* L. species/varieties in eastern Yunnan based on genetic similarity coefficients NJ method

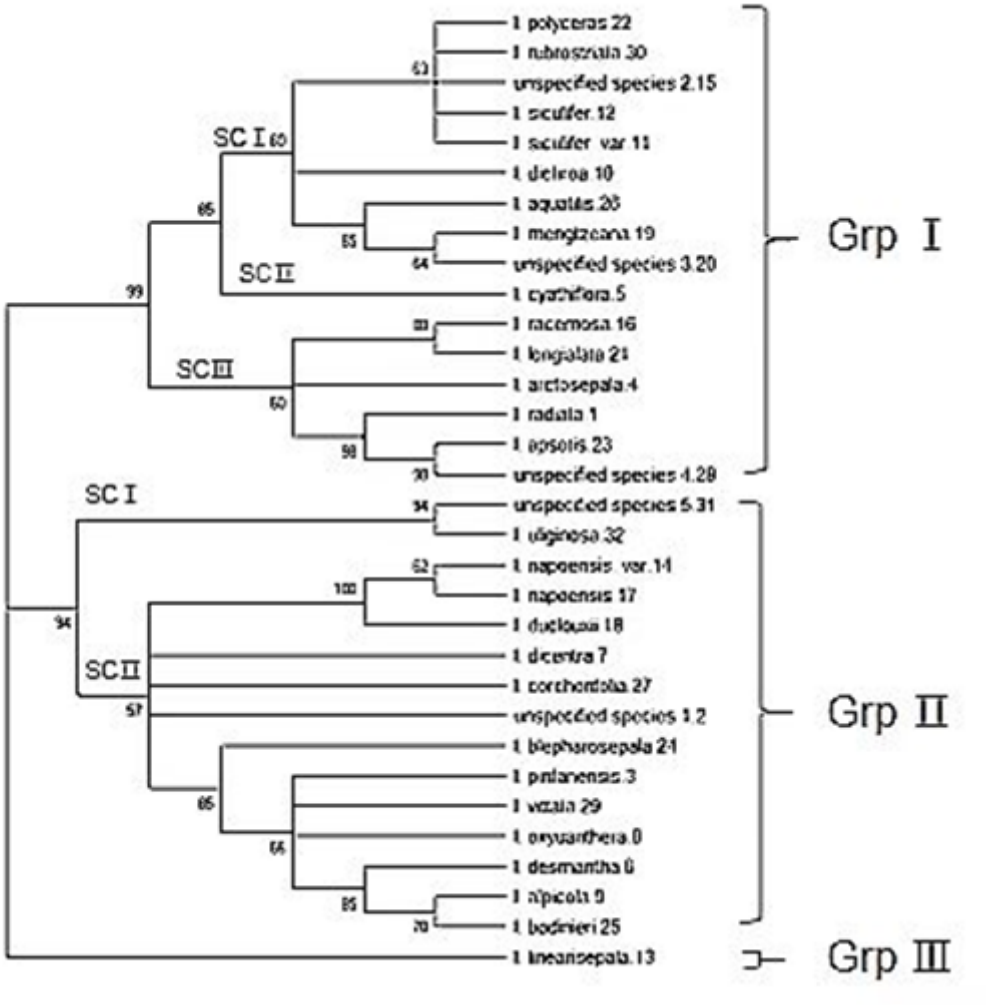

Figure 3

Phylogenetic tree based on 32 species/varieties of Impatiens in eastern Yunnan *atpB-rbcL* sequence (NJ method)

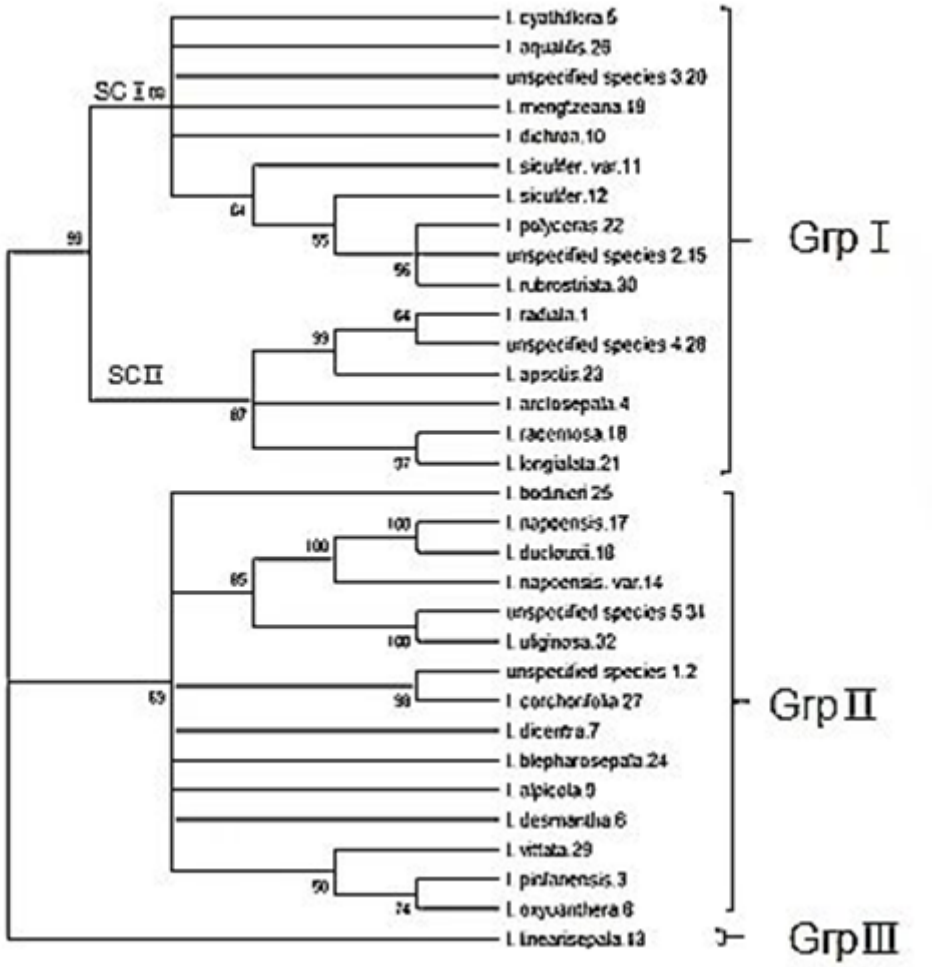

Figure 4

Phylogenetic tree based on 32 species/varieties of Impatiens in eastern Yunnan *trnL-F* sequence (NJ method)

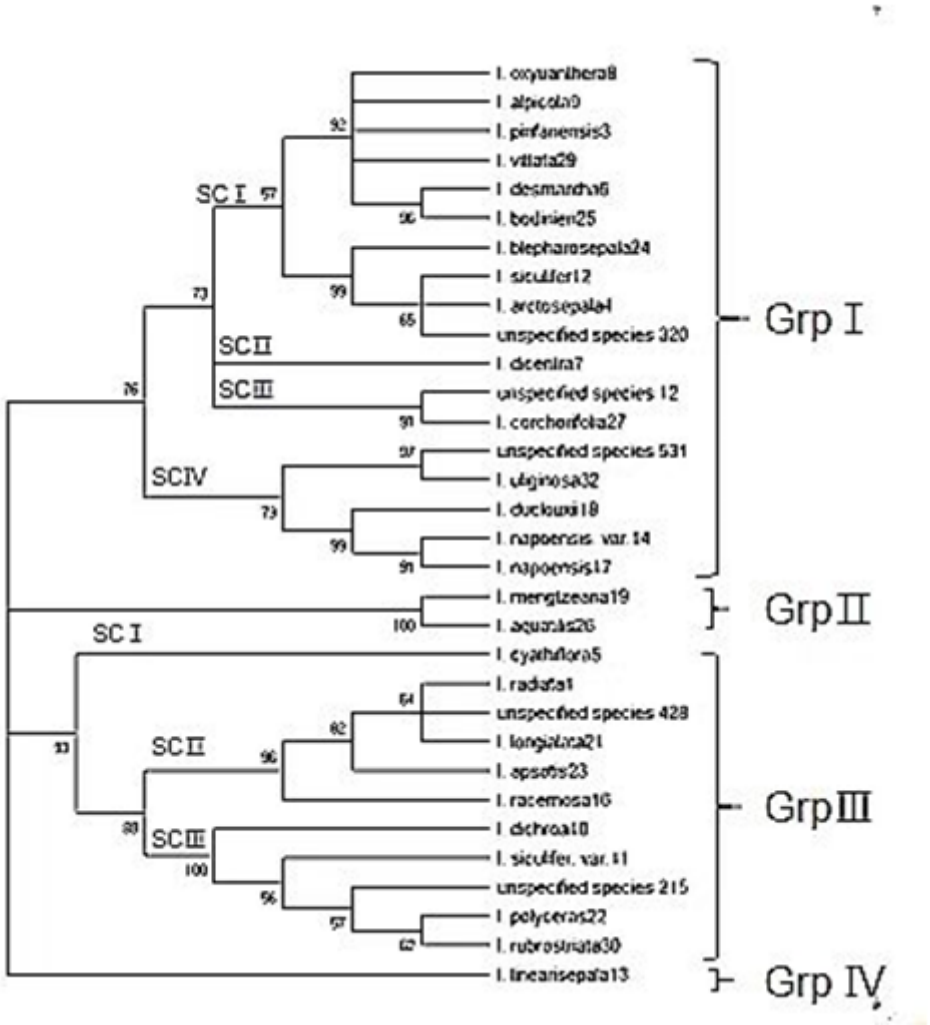

Figure 5

Phylogenetic tree based on32 species/varieties of Impatiens in eastern Yunnan *DEF* gene sequence (NJ method)