Australian Journal of Crop Science AJCS

AJCS 16(04):471-478 (2022) ISSN:1835-2707
doi: 10.21475/ajcs.22.16.04.p3421

Morphological characteristics and DNA barcoding in bach hop (*Lilium poilanei* Gagnep) in Vietnam

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Abstract

The *Lilium* genus is a member of the *Liliaceae* family and is comprised of approximately 110 to 120 species. This genus is considered the largest genome with a haploid DNA content in the plant kingdom and is currently receiving much attention for its great commercial and trading prospects. Among them, *Lilium poilanei* belongs to the Sinomartagon group, plays an important role in the breeding of Asiatic hybrids. *L. poilanei,* widely known as an endemic plant in mountainous areas of Sapa, Vietnam and has been intensively exploited due to great commercial significance. In this study, the morphological characteristics and DNA sequence data including the nuclear ribosomal DNA segments of ITS, ITS2, and plastid *mat*K*, psbA-trn*H and *rbc*L, *rpo*C1 were applied to identify *L. poilanei* collected. The results showed that some morphological details of the lily were specifically analyzed and both markers ITS2 and *rpo*C1 disclosed superiority in discrimination of *L. polinanei* with 100% similarity score by the one of ours reported in NCBI Genebank with the accession number of KR632775.1 and KR632777.1, respectively, while the remaining regions have been neither unamplified nor unsuccessfully discriminated and identified. Our findings in morphology and barcodes were reliable, effective and powerful for distinguishing *L. poilanei* and may identify other species of the family of *Liliaceae*.

Keywords: Accession Number, Genbank, *Lilium poilanei Gagnep.*, Sapa flower, *rpo*C1, ITS2.
Abbreviations: ITS_Internal Transcribed Spacer; DNA_Deoxyribonucleic acid; NCBI_National Central for Biotechnology Information; PCR_Polymerase Chain Reaction.

Introduction

The genus of *Lilium* comprises approximately 110 to 120 species and is considered the largest genomes which are distributed across the cold and temperate regions in the northern hemisphere (Liang and Minoru, 2000). *Lilium* species including hybrids and the decoratively cultivated varieties are grown due to their esthetic value and medicinal properties (Du et al., 2017) and currently received much attention for their great commercial prospects. Lilies were horticulturally classified in division 9 and 8 sub-divisions, of which *L. poilanei* belongs to Sinomartagon and was important for breeding Asiatic hybrid (Comber, 1949). In some recent years, the Lilium genus has been facing a severe threat of genetic erosions of some endangered plant species because of overexploitation, habitat fragmentation, pests and disease infestations and climate change (Dhiman et al., 2021). In the fact that *L.poilanei* was discovered in Sapa, Lao Cai province in Vietnam, and ranked as a rare and endangered species (Tuyl et al., 2011; Averyanov et al., 2016). The local name in Vietnamese of *L. poilanei* is “Bách Hợp”, which means the hundreds of combinations in one. However, the species are facing major threats like a decrease in habitats through frequent and rapid colonization of wild plants and overexploitation and adverse impacts from global climate change (Thin, 1998; Bradshaw et al., 2009).

Conventionally, the identification of taxonomic species has been certain of the morphological characteristics of plant species and experienced taxonomists. However, in the last decades, based on the DNA sequences of short, standardized gene fragments, DNA barcodes have been applied for species discrimination as promising molecular tools (Fišer and Buzan, 2014; Zhang et al., 2015). It is estimated that approximately millions of plant species have been yearly described taxonomically (Vernooy et al., 2010). DNA barcode, a time-saving, highly variable, and standard approach to identifying species through the comparison of DNA sequence database has been used to identify both unknown and known species (Duong et al., 2018; Maia et al., 2012). However, the haploid DNA content of *Lilium* species ranges from 32.75 to 47.90 pg which made it arduous for molecular marker performance (Tuyl, 2017). The biotechnological applications including molecular markers, tissue culture and recombinant DNA technology have the key role in development of *Lilium* varieties with enhanced traits. Among them, ITS regions have already been used for the classification of *Lilium* genus (Nishikawa et al., 1999; Nishikawa, 2007; Chen et al., 2010;

Sultana et al., 2011; Ayam, 2013; Zheng et al., 2014). Nevertheless, molecular application of barcoding in the genus *Lilium* has been performed on a limited scale (Nishikawa, 2001). To the best of our knowledge, very sporadic studies have been reported the *Lilium* population and availability of the taxonomic characteristic genomic sequence of the endemic lilies in Vietnam, of which the preliminary reports documented a few morphological identifications of this plant (Gagnepain, 1934 and Thao et al., 2009). Therefore, immediate approaches and sustainable strategies such as biodiversity surveys and biological conservation should be simultaneously applied by using both traditional methods and modern methods for quicker identification of this species. Hence, our attempts in this study were to describe the morphological characteristics together and apply DNA barcodes for further accurate discrimination of this plant.

Results

Morphological characteristics and habitats of L. poinlanei

The lily plant (*L.poinlanei*) was once claimed to be endemic to the moist, rainy, cold, high-altitude mountains of Sapa district, Lao Cai province, Vietnam. Sapa is a rural, mountainous district and is 1500m above sea level, located at 22°7' to 22°28'46''0N latitude and 103°43'28'' to 104°04'15''0E longitude (Fig1, Fig2). This region typically has a temperate climate (cool in summers and cold in winters). The average temperature of Sapa is about 15-16°C and the average rainfall is from 2800 to 3400 mm, annually. In this study, we found that in every 100 m^2, there were 3 to 5 plants on Ham Rong mountain (Sampling area 1. Fig 1), 10 to 20 plants on the way from Sapa's Mau Thuong temple to O Qui Ho village to Ban Khoang village (Sampling areas 2 and 3. Fig 1), and 30 to 50 plants on the western side of Sapa on the way 4D route (Sampling area 4. Fig1). Around the lily in natural conditions, 12 other plant species belonging to the family of *Poaceae, Cyperaceae, Zingiberaceae, Hypoxidaceae, Melastomataceae, Ranunculaceae, Geraniaceae,* and *Smilacaceae* are regularly found with the lily plant. Of which, *Sasa japonica, Sinarundinaria griffithiana,* and *Arundinaria amabilis* (family of *Poaceae*), are the most dominant growing nearby the *L. poilanei* (data not shown). Their local names and scientific names are illustrated in Table 1 and Fig 3.

Some morphological characteristics of the lily plant are presented in Tables 2,3,4 and Fig 4. The lily bulbs collected after the flowering season (Fig 4 a,b, Table 2) had an average bulb diameter of 6.93 ± 0.99 to 7.85 ± 1.23 cm, and the average height ranged from 5.07 ± 0.69 to 5.78 ± 0.78 (cm). Moreover, the average weight of the purple scale bulbs was 161.12 ± 21.39 (g), while the smaller yellowish-white bulbs were 103.18 ± 13.87(g), respectively. Their bulbs with broadly globose shapes a different bulb scales were likely to be loose or tight. Bulb differences associated with other plant features such as the leaves and flowers are described in Tables 2, 3 and Table 4. This plant had a big number of leaves, approximately 120.6 ± 7.15 pieces in white bulb plants to 150.08 ± 17.44 in purple bulb plants. The leaf's length and width of lily plant with light yellow bulbs ranged from 12.52 ± 1.21; 2.77 ± 0.35 cm, and of purple bulbs 13.65 ± 1.72; 2.9 ± 0.57 cm, respectively. The leaves were green, linear, and narrowly obovate - lanceolate and gradually tapering. The lily leaves were straight, glabrous, and green with scattered leaves position as presented some detailed lily flowers in Table 3. It is a stalk-bearing flower with a long flower peduncle, from 7.41±1.09 to 8.41±1.07 cm. The length of the stamens was not so much shorter than that of pistils. In yellowish-white, light yellow bulbs' flowers, the length of the stamens was 6.13 ± 0.59 (cm), while the pistil length was 6.95 ± 0.39 (cm); and the purple bulb plants had stamen length of 6.93 ± 0.33 (cm) and pistil length of 7.76 ± 1.1(cm).

The lily starts to flower annually at the end of June to the middle of August. The lily shape is a long tubular shape with a slender tube gradually expanding toward the apex. The inflorescence is a raceme, two or three flowers on each pedicel with extreme fragrance. The flowers are horizontal and slightly descending. Tepals, petals recurved the apical, slightly revolute, and with two kinds of color. The first is brownish yellow petals with the purple bulbs, and the second was the light green petals with light-yellow bulbs. Especially, in the insides of the petals, there are several dark red spots concentrated from the middle to lower points (Fig 4). Besides that, all organs of the plant have morphological characteristics related to each other. For example, in this research, when the weight of the bulb was approximately 100 g to 160 g, the traits of the leaves and flowers were slightly changed as shown in Table 2 and Table 4, and the heigh of the plant was around 1 to 1.2 meter.

The molecular base of L. poilanei Gapnep

In this study, the ITS1/2, and plastid *mat*K, *psb*A-*trn*H, *rpo*C1 and *rbc*L were used and assessed as potential *Lilium* barcoding regions. Unfortunately, while *mat*K, *psb*A-*trn*H, and *rbc*L were attempted with the sets of standard primers, data obtained *mat*K were either unamplified, unclear or resultless. Hence, those marker regions were subsequently eliminated (data not shown). The other markers *psb*A-*trn*H and *rbc*L were adequately amplified by applying the standard pairs and PCR protocols, but the results attained from the sequence analyses were not sufficient variation to be germane for barcoding the genus of *Lilium*. Contrarily, measurement of DNA sequence alignment for *L. poilanei* for *rpo*C1 and ITS1/2 markers in nucleotide BLAST showed a 100% similarity score with the one reported in NCBI, GeneBank. There was no sequence report for the *rpo*C1 gene and therefore, one was deposited as a new sequence submission by direct submission to NCBI and was assigned with the accession number KC539824. The sequence results for both *rpo*C1 and ITS1/2 were comparatively inferred. The deposit of the two new marker sequences to GeneBank is for open access and public use on their phylogeny. The nucleotide sequences of these two markers - genes were submitted on NCBI as KR632775.1 and KR632777.1 for ITS1/2 and *rpo*C1, respectively.

Moreover, the diversity level of our species was compared with other *Lilium* species, and three species belong to *Fritillaria* genus as an outgroup using the ITS1/2 and rpoC1 sequences (Fig. 5). The result illustrates that the ITS1/2 sequence of *L.poilanei* (KR632775.1) shared only from 91.9 to 92% identity with three *Fritillaria* species (MW025086.1, MW025087, MW025100), while showing the closer relationship with other *Lilium* species with identities ranging from 93.3 to 96.3%. This result reveals that *Lilium poilanei* belongs to *Lilium* genus, whereas it is reported the low identity in ITS1/2 region with other *Lilium* species (Fig. 5A). Furthermore, the identity of *rpo*C1 sequence of *Lilium poilanei* (KR632777.1) reached 99.1% and 99.8% with *Fritillaria* and *Lilium* species, respectively. This result showed the *rpo*C1 is a conserved sequence between each genus belonging to the Liliaceae family. However, *rpo*C1 sequence of *L. poilanei* shared a closer identity within *Lilium* genus compared to *Fritillaria* genus (Fig. 5B). In conclusion, both ITS1/2 and *rpo*C1 showed the effective identification of DNA barcoding, whereas ITS1/2 disclosed the more specific for DNA barcoding.

Table 1. Associated plants with the lily.

Number	Local name	Scientific name	Family
1.	Trúc đũa	*Sasa japonica*	Poaceae
2.	Sặt gai vòng	*Sinarundinaria griffithiana*	Poaceae
3.	Trúc thưa	*Arundinaria amabilis*	Poaceae
4.	Sặt Fansipan	*Arundinaria petelotii*	Poaceae
5.	Cói ấn	*Carex indica*	Cyperaceae
6.	Cói túi	*Carex alopecuroides*	Cyperaceae
7.	Ngải tiên	*Hedychium flavum*	Zingiberaceae
8.	Sâm cau	*Curculigo crassifolia*	Hypoxidaceae
9.	Sắc tử chùm tụ tán	*Oxyspora paniculata*	Melastomataceae
10.	Hoàng liên chân gà	*Coptis quinquesecta*	Ranunculaceae
11.	Mỏ hạc	*Geranium homeanum*	Geraniaceae
12.	Kim cang petelot	*Smilax petelotii*	Smilacaceae

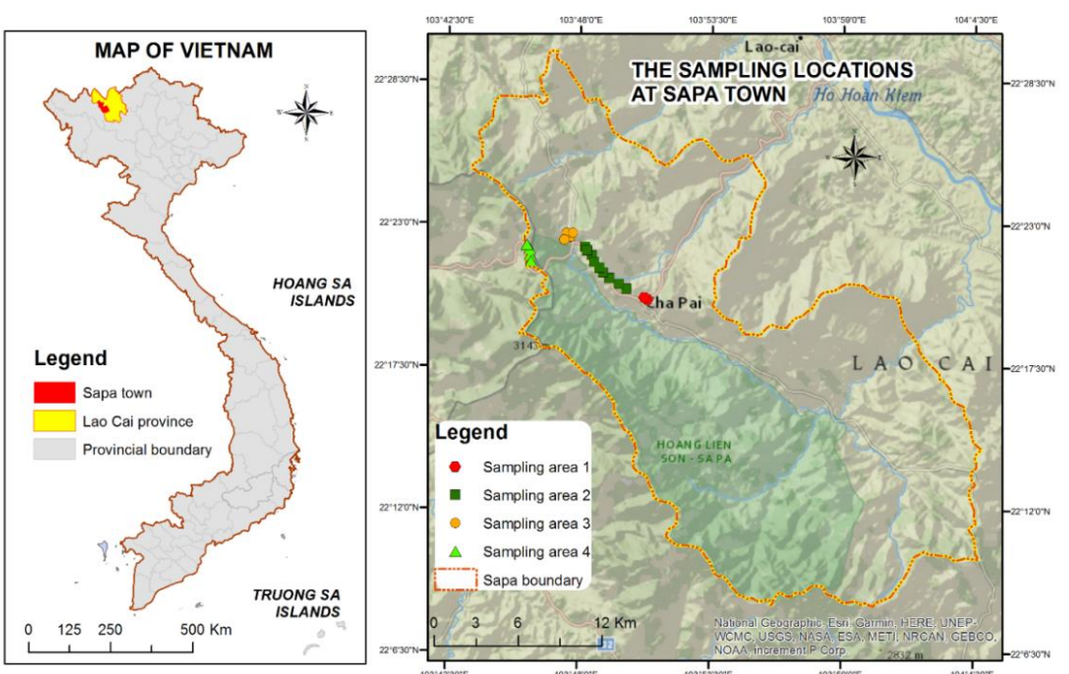

Figure 1. Map of the sampling sites. The map was produced using ArcGIS 10.3. Sampling area 1 was at Ham Rong mountain; The sampling area 2 ranged from Sapa's Mau Thuong temple to O Qui Ho village; The sampling area 3 was along with Ban Khoang village; The sampling area 4 located on the western border of the town.

Table 2. Several morphological characteristics of the lily bulb.

Bulbs' characteristic	Lily plant with light yellow bulbs	Lily plants with purple bulbs
Color	Light yellow	Purple
Bulb's width (cm)	6.93 ± 0.99	7.85 ± 1.23
Bulb height (cm)	5.07 ± 0.69	5.78 ± 0.78

Figure 2. Scenery of habitats of *L. poilanei* in Sapa, Lao Cai, Vietnam. Photos were taken by Nguyen Huu Cuong.

Table 3. Several morphological characteristics of the lily leaves.

Leaves' characteristic	Lily plant with light yellow bulbs	Lily plants with purple bulbs
Number of leaves (piece)	120.6 ± 7.15	150.08 ± 17.44
Leaf 's length (cm)	12.52 ± 1.21	13.65 ± 1.72
Leaf's width (cm)	2.77 ± 0.35	2.9 ± 0.57

Figure 3. *L. poilanei* and its associated wild plants. Photos were taken by Nguyen Huu Cuong.

Table 4. Several morphological characteristics of the lily flowers.

Flower's characteristic	Lily plants with light yellow bulbs	Lily plant with purple bulbs
Flower peduncle length (cm)	7.41 ± 1.09	8.41 ± 1.07
Petal length (cm)	10.49 ± 0.86	11.16 ± 0.81
Petal width (cm)	2.04 ± 0.29	2.13 ± 0.18
Stamen length (cm)	6.13 ± 0.59	6 .93 ± 0.33
Anther length (cm)	2.06 ± 0.18	2.03 ± 0.22
Anther width (cm)	0.26 ± 0.03	0.27 ± 0.04
Diameter of pistil (cm)	0.16 ± 0.02	0.28 ± 0.03
Pistil length (cm)	6.95 ± 0.39	7.76 ± 1.10

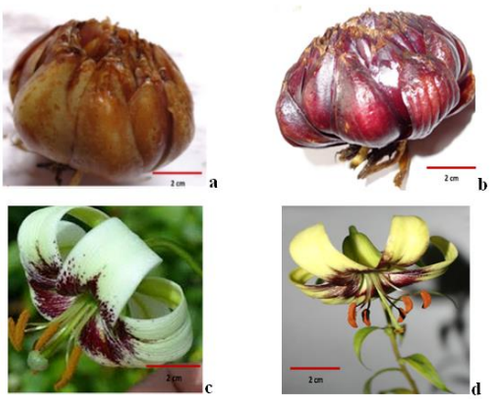

Figure 4. Yellowish-white bulb (a); Purple bulb (b); and flower of plant with light yellow bulbs (c); and flower of plant with purple bulbs (d).

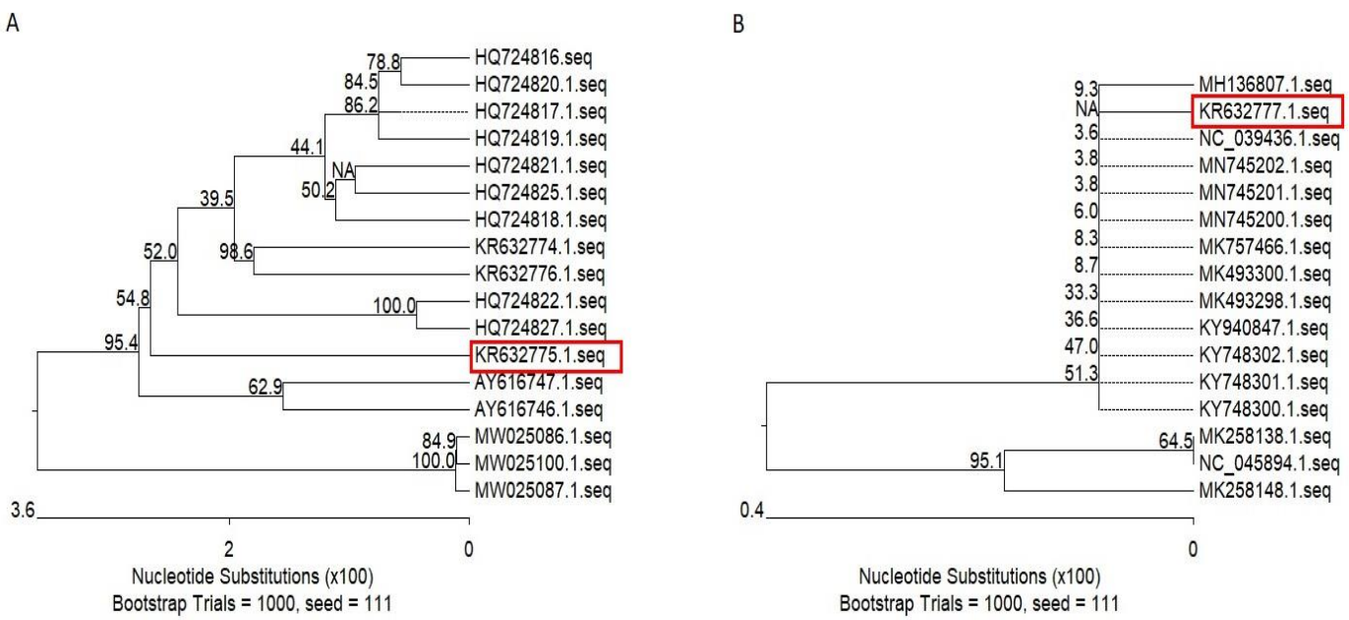

Figure. 5. The phylogenetic tree based on the alignment of the ITS1/2 (A) and *rpoC1* (B) regions of *L.poilanei,* and other *Lilium* species, or three *Fritillaria* species.

Discussion

As indicated above, the lily with a beautiful flower with the light yellow or light green and a sweet aroma was discovered and is available in Sapa, Lao Cai province in Vietnam (Fig 4 c, d). However, it has been severely declining due to the arbitrary and overexploitation and lack of a sustainable approach to conservation. Originally, the lily was assessed some main morphological characteristics as *L. poilanei* and has been ranked as one of the rare and endangered species in the world (Thao et al., 2009; Averyanov et al., 2016). Moreover, as major lineages of the genus *Lilium* and belonging to Sinomartagon, *L. poilanei* was evaluated as a key line for breeding Asiatic hybrids. Therefore, in this study, it is noteworthy to critically narrate this plant with more detailed morphological characteristics, its habitats, and the surrounding natural environment of this plant in natural conditions. Our current investigation of the lily in the cited studies found a very limited number of its distribution in Sapa district, Lao Cai province (Fig 1). Some specific locations where the lily could only be discovered on cliffs at various positions where were ranged from 5 to 40 m high, places such as Ham Rong mountain, the mountains next to Sapa's Den Mau Thuong temple, some mountains being one side away from Ban Khoang village, 4D route to Lai Chau province (Fig 2). The average height of the lily in our investigation was lower than the previous report of Averyanov et al. (2016) who documented that the lily with an erect stout stem to 2 m tall. Therefore, the habitats of *Lilium* are at risk to deteriorate. As our investigation, *L.poilanei* species is found in wild habitats in Sapa areas, therefore its distribution may be mainly based on environmental conditions such as temperature, altitude, light intensity and humidity, etc.

Currently, in Sapa district, it is easy to find *Sasa japonica, S. griffithiana, A. amabilis,* and other plants (Table 1, Fig 3) growing around the lily plant. These invasive plants grow vigorously and directly compete and encroach on the growth and development of the lily due to the allelopathic effects (Favaretto et al., 2018; Khanh et al., 2007) and are also one of the main causes of the decline in the lily individuals and habitats in these areas. Moreover, Thin and Thoi (1998) declared that the competitive two plants with the lily, common on karstic rocky limestone, on very steep rocky slopes, and shelves of shady cliffs near mountain tops were *Berberin wallichiana* and *Oxyspora paniculata*.

In this study, we found that there have been some typical morphological characteristics of *L. poilanei* such as a high number of leaves with stalkless, especially, the number of leaves in purple bulb plants was higher than white bulb plants (Tables 2, 3, 4, and Fig 4). Our results were consistent with the report of Gagnepain 1934, included 6 petals, rolling outwards, and surrounding the sparkle red inner cylinder about 2 cm. All stamens are smooth and carry rice - grain - shape anthers with 12 mm in length in which the pollen grains are yellow, long, and vertical. Besides, there has been one pistil with a stigma of three lobes (Gagnepain, 1934). Additionally, the structure of the stamen and pistil indicate that the plant is suitable for self-pollination. The anthers were large and had many pollen grains, which is an important criterion for breeding.

In the lily family, according to the classification of Comber (1949), the genus *Lilium* consists of seven sections including *Martagon*, *Pseudolirium*, *Liriotypus, Archelirion, Sinomartagon, Leucolirion,* and *Daurolirion*. Then, based on not only morphological taxonomy, but also molecular phylogenetic methods, some phylogenetic clades of *Lilium* have been more understandable, and the updated system classifies the genus into seven sections. ITS of nuclear ribosomal sequence was firstly used to build some phylogenetic studies of genus *Lilium* a long time ago (Nishikawa et al., 1999; Nishikawa et al., 2001). After that, some species have been identified by exploiting ITS regions (Rešetnik et al., 2007; Sultana et al., 2011; Du et al., 2014; Kim et al., 2019) and mitochondrial DNA markers such as *psb*A-*trn*H, *mat*K, *rbc*L, *rpo*C1, *ycf*5. Unfortunately, these sequences, ITS, ITS2, *psb*A-*trn*H, *mat*K, and *rbc*L, etc. were used to analyze in terms of variation of inter-and intra-species of some lily varieties. The barcoding sequences and a neighbor-joining tree were gained to distinguish the genus of *Lilium* based on the attained sequences (Zheng et al., 2014). Recently, chloroplast genomes of some lilies were also sequenced and used for comparative and phylogenetic analyses (Du et al., 2017) and useful for plastid genome region studies including *mat*K, *rbc*L, *ndh*F, and spacer regions of *trn*L-F, *rpl32*-*trn*L, *trn*H-*psb*A for *Lilium* molecular systematic analysis. However, there have been existed an incongruence finding between plastid and nuclear which also were reported in several studies of some other genera because of insufficient phylogenetic signals, incomplete lineage sorting, or complex evolutionary issues (Patterson and Givnish, 2002; Hayashi and Kawano, 2000; Gao et al., 2013).

In terms of DNA barcoding in the identification of plant species, numerous studies have used the internal transcribed spacers (ITS) of the nucleus DNA ribosome (rDNA) makers to contribute to the processes of plant species identification and discrimination (Erickson et al., 2008; Vijayan and Tsou, 2010). However, some cryptic and endemic plant species with complex groups and morphological characters are challenging to identify by molecular markers (Tuyl et al., 2011). Therefore, our attempts in this study were to search for the optimal markers for the identification of *L. poilanei*. Indeed, no marker disclosed all desired characteristics required for plant barcoding is available yet. However, some reports have suggested simultaneously applying at least more than two markers for accurate identification (Pečnikar and Buzan, 2014; Tuong et al., 2020). In this study, the DNA barcode primers including: ITS, ITS2, and plastid *mat*K, *psbA*-*trn*H and *rbc*L, *rpo*C1 regions were used. Unfortunately, the ITS, *mat*K, *psb*A-*trn*H, and *rbc*L were not suitably applied for barcoding of the *L. poilanei* due to either unamplified DNA PCR or insufficient variation for barcoding. Hence, those markers were eliminated in this study. In our finding, only ITS2 and *rpo*C1 markers resulted in the clearly informatic data of *L. poilanei*, while other markers were not shown any valuable data. Strikingly, the nucleotide sequences of *rpo*C1 and ITS2 of *L. poilanei* have not been reported by any elsewhere studies to any GeneBank. As indicated above, our results are agreeable with the report of Ayam (2013) who documented that the *rbc*L mitochondrial gene was determined to be more reliable for *L. mackliniae* than the *mat*K gene and the ITS2 gene was suggested to serve as a common barcode for identifying a wider range of plant species (Chen et al., 2010).

In summary, our report suggests that DNA segments of *rpo*C1 and ITS2 were an effective barcoding tool for prompt and accurate taxonomic analyses of a cryptic species of *L. poilanei* in Vietnam. Our findings have provided useful information on the morphological traits and effective barcoding to further breeding, utilization, and conservation of the *Lilium* genetic resources.

Materials and Methods

Plant materials

L.poilanei was sampled from the high mountain areas such as in O Qui Ho, Ham Rong, Ban Khoang of Sapa, Lao Cai province, Vietnam. The detailed information of sampling areas is presented in Fig.1.

Morphological evaluation

Several major morphological characteristics of the lily were investigated using the method of Wu et al. (2014). Some bulb parameters were analyzed based on the main morphological traits such as bulb diameter, average height, average weight, number of leaves, length of leaves. Moreover, some other characteristics of flowers as length and width of tepals, flower color, stamens length, length and width of the anther, diameter and length of the pistil, flower peduncle length were also recorded for data statistics. The sampling sites were collected and analyzed using ArcGIS 10.3 and Excel version 2016.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from fresh leaves of lily following the protocol of Grattapaglia and Sederoff (1994). Polymerase chain reaction (PCR) of the regions was carried out in a Mastercycler personal 120V (Eppendorf, USA) using 1µl of genomic DNA 100 ng/µl as a template in a 25 µl reaction mixture including Master mix dNTPs Tag DNA polymerase and forward and reverse primers (10 pM). DNA barcode primers were used with sequences of ITS2 gene and RNA polymerase C (*rpo*C1) gene. DNA barcode primers were ITS1/2 F: ACGAATTCATGGTCCGGTGAAGTGTTCG; R: TAGAATTCCCC-GGTTCGCTCGCCGTTAC, and plastid *mat*K F: ATCCATCTGGAAAT-CTTAGTTC; R: CTTCCTCTGTAAAGAATTC, *psb*A-*trn*H F: GTTATG-CATGAACGTAATGCTC; R: CGCGCATGGTGGATTCACAATCC, *rpo*C1 F: GTGGATACACTTCTTGATAATGG, R: TGAGAAAACATAAGTAAACGGGC (Deka, 2020) and *rbc*L F: ATGTCACCACAAACAGAAAC; R: TCGC-ATGTACCTGCAGTAGC (Yu et al., 2016). PCR analyses for ITS1/2, *mat*K, *psb*A-*trn*H and *rbc*L barcoding regions were conducted following the method of Madesis et al (2012). The PCR products were performed on a 1.0% agarose gel in 0.5XTBE buffer and analyzed by GelDoc and Quantity One (Bio-rad) and then purified with Kit QIAquick Gel Extraction (QIAGEN). The purified PCR products of the genes of the samples were then sequenced by Sanger dideoxy sequencing technology using ABI PRISM 3100 Avant Genetic Analyzer with the kit of BigDye Terminator v. 3.2 Cycle Sequencing (Macrogen Inc. Korea).

Sequence alignment and data analysis

These DNA sequences were analyzed the diversity level using DNASTAR Lasergene v 7.1.0 with bootstrap 1000 and seed 111.

These DNA sequences were blasted on NCBI using Nucleotide BLAST to confirm whether they are available in the GeneBank or to make a new identification submission and check the best BLAST hit for the query sequence.

Conclusions

In conclusion, *L. poilanei,* an endemic lily in Sapa, Lao Cai province, Vietnam, had been assessed their morphological characteristics and potential growth areas in Sapa and their associated wild plants. DNA barcodes of both ITS2 and *rpo*C1 primers disclosed superiority in discrimination of *L. polinanei* with a high similarity score with the accession number of KR632775.1 and KR632777.1 newly registered in NCBI Genebank. These detailed data of the species could contribute to further sustainable development of lily breeding strategies, and contribute to biodiversity surveys and biological conservations of the rare, valuable lilies.

Acknowledgments

Special thanks to the local persons for their help in plant collection, thanks to Vietnam National University of Agriculture for funding the research and Biotechnology Institutes for facilitating some equipment and the partial chemical during the study.

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