Lilium spp. pollen in China (Liliaceae): Taxonomic and Phylogenetic Implications and Pollen Evolution Related to Environmental Conditions

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Introduction

Approximately 110 to 115 Lilium species are distributed in the cold and temperate regions of the Northern Hemisphere [1,2], particularly in East Asia, the Himalayas and Hengduan Mountains, North America and Europe. A total of 55 species occur in China [2]. De Jong [3] and Patterson and Givnish [4] consider southwest China and the Himalayas to be the center of origin of Lilium. De Jong [3] and Patterson and Givnish [4] consider southwest China and the Himalayas to be the center of origin of Lilium. Classification of this genus has been historically complicated. Several classifications for Lilium have been proposed based on morphological characters. Detailed studies have been performed for East Asia, European and North American Lilium species [5–7]. Based on 13 morphological characters and two germination types, Comber divided this genus into the following seven sections: Martagon Rchb., Pseudolirium Endl. which is limited to North America, Liriotypus Asch. and Graeb, which is distributed across Europe and the Caucasus, Archelirion Baker, Sinomartagon Comber, Lophophorum Wilson, and Daurolirion Comber, representing the most widely accepted taxonomical divisions [8]. Wang and Tang recognized sect. Lophophorum (Bur. et Franch.) Wang et Tang out of sect. Sinomartagon Comber. Chinese species were divided into five sections: Martagon, Archelirion, Sinomartagon, Lopholirion and Lophophorum.

Recently, molecular phylogenetic analyses and chromosome techniques have improved the understanding of several groups within the genus and modified the phylogenetic position of Comber’s classification, such as placement of sect. Daurolirion Comber in sect. Sinomartagon, L. henryi in subsect. Leucolirion 6b, modification of sect. Lophophorum and relationship confirmation in sect. Liriotypus [12–13,16–21]. Preliminary research found that sect. Sinomartagon, which mainly occurred in China, was complicated and polyphyletic. As indicated by Patterson and Givnish [4], intercontinental dispersal details of the genus Lilium are not yet clear. The division of subsect. Sinomartagon 5c and sect. Lophophorum are controversial and will require further research. Nishikawa et al. [18,19] suggested that L. henryi be classified into subsect. Leucolirion 6a and that it showed similar morphological features with L. rosthornii, thereby demonstrating that the phylogenetic position of L. rosthornii needs further study.

There are few relevant studies regarding pollen morphology which defines the taxonomic and reflect the evolution of the genus. According to the description within Lilium by Baranova [22] based on the number, shape and arrangement of columellae that form the muri, there are three morphological types of pollen: (1) Martagon (muri formed by rectangular columellae); (2) Callose

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(muri formed by rounded columellae); and (3) Concolor (muri formed by separated rounded and polygonal columellae). Previous studies found that most Lilium species have single pollen grains. However, pollen tetrads were found in *L. sempervirens* var. H. Lév. and *L. amorum* E. H. Wilson ex Sealy, and the size and sculptural elements confirmed the taxa as two subspecies in *L. sempervirens* [23]. The pollen morphology of *L. laetiflorum* (Bur. et Franch.) Franch., *L. henrii* Franch., *L. souliei* Franch., Scaly and *L. nanum* Klotz. et Garcke supported placement in Lilium, which differs from *Nonocharis* in aperture and sculptural elements, and showed an evolutionary apertural trend from monocolpate to porate [24]. Muratovíc et al. [25] showed that two related European species, *L. bontanicum* and *L. carniolicum*, share similar pollen morphology. In addition, pollen morphology of some Chinese species under scanning electron microscopy (SEM), including 9 species described by Li and Qin [26], 10 species and 3 cultivars by Zhang et al. [27], and 12 species and 6 cultivars by Wu et al. [28], could provide taxonomic implications within *Lilium*; pollen has not only the commonness of genus, but also the specificity of single species. Interspecific pollen size and morphological characteristics has some difference, which has a certain reference value for the classification of *Lilium*. For example, there are differences in pollen size among *L. cernuum*, *L. lancifolium* and *L. pumilum*. Also there are significant differences in pollen ornamentation and morphology between *L. lancifolium* and its variety *L. lancifolium* var. *crisiformium* from Qingning Mountains. Results obtained by Wang et al. [29] and Liu [30] indicate that pollen size parameters of *L. pumilum* and *L. concolor* from different provenances showed different degrees of variation. Determining whether this is a universal phenomenon in other species or if it is an important characteristic of species from different provenances requires a large amount of data. Therefore, a comprehensive study of *Lilium* pollen morphology in China, particularly a comparative study between populations or provenances using SEM, was needed. Environmental constraints act on patterns of differentiation, as all character states are the consequence of interactions between phylogenetic and environmental constraints [31]. Pollen could be considered a functional unit, with the exine structure as a compromise between four main functions [32]: protective, harsmomegaetic (the ability to absorb bending stresses, as occur during desiccation), reservoir (the role in functions [32]: protective, harmomegathic (the ability to absorb

Materials and Methods

Ethics Statement

For conservation reasons, we collected only small amounts of *Lilium* pollen. Our activities did not have any adverse effects of *Lilium* populations, and permission for collecting was obtained from National Engineering Research Center for Flowers of China (No.35 Tsinghua East Road Haidian District, Beijing, P.R. China). Table 1 shows the origin of all species studied which were collected fresh. Our observations for 21 taxa represented by 49 samples were also included in the database. Division into the sections was based on studies by Comber [8], Gao et al. [13], Nishikawa et al. [18,19] and Du et al. [16] as follows: two species and one variety of sect. *Martagon*, nine species and three varieties of sect. *Simaraginum* including *L. drauricum* Ker-Gawl. (sect. *Dauroterion* Comber), eight species and one variety of sect. *Leucolirion* including *L. brownii* F. E. Brown ex Mielcz. (sect. *Archelirion* Comber), *L. henryi* Baker and *L. rosthornii* Diels (sect. *Simaraginum* Comber), one species of sect. *Archelirion*, two species of sect. *Liritum*, three species of sect. *Leucolirion*, and six species and one variety of sect. *Laphophorum* (Table 1).

To compare pollen features at the genus level, a database with results of the present study and those of Halbritter [34], Kosenko [35], Liang et al. [23,24], Muratovíc et al. [25], Wu et al. [28], Zhang et al. [27] and Zhang et al. [36] was established (Table 1). Because the results do not permit a calculation of all the parameters cited above, our observations from 49 samples representing 21 taxa was used only for statistical analyses.

Morphological observations were conducted using SEM. Pollen was directly attached to double-sided adhesive tape and examined under the microscope to locate the pollen. The sample was then taped to the object stage. Following gold spray coating, observation and image acquisition were conducted using a Hitachi S-3400 scanning electron microscope following the method described by Avetissian [37]. All microscopy procedures were performed at the Biotechnology Centre, Beijing Forestry University, China.

Biometric measurements were made using Image-Pro Plus 6.0 (Media Cybernetics, USA). For each sample, 10 (3 for *L. brownii* from Shennongjia, Hubei Province) fully developed pollen grains were measured. Parameters considered were the polar axis (P), equatorial diameter (E), P/E ratio, lumina diameter and muri width (Table 1). The description of pollen morphology was based on the shape and sculpturing classifications of Baranova [22]. Arithmetic mean and standard deviations are shown for each parameter.

To investigate pollen evolution, pollen features from the database were superimposed onto a phylogenetic framework constructed by internal transcribed spacer (ITS) sequences. We selected 23 taxa, including *Cordosporum giganteum* as an outgroup based on preliminary study [16]. The other 16 taxa, including an outgroup of *Notobolithus bulliformis*, were cited [13,19–20]. All of the sequence data from the ITS regions were double-checked visually, edited in BioEdit 5.0.6 [30], and aligned in Clustal X 1.83 [39] using default settings. Phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML). The MP tree was constructed with PAUP* 4.0b10 [40]. ML phylogenetic analysis was performed using RAxML v. 7.0.4 with unique model parameters for ITS sequences [41]. A general time reversible model (GTR) was applied with discrete gamma distribution. Bootstrap pseudo replicates were performed 1000 times using the fast bootstrapping option and the best scoring ML tree. Phylogenetic trees were visualized using Treeview [42]. The best scoring tree was visualized with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/).

SPSS 10.0 was used for statistical analyses. One-way analysis of variance (ANOVA) was used to evaluate whether or not differences in pollen characteristics from different provenances were significant. In those cases in which ANOVA revealed significant differences, a least significant difference (LSD) test was performed (Table 2). Correlation analysis was used to evaluate the
Table 1. Origin of the material and numerical results of the study.

| Samples                  | Voucher                          | P (μm) *  | E (μm) # | P/E | Lumina (μm) | Muri width (μm) | Pollen type** |
|--------------------------|----------------------------------|-----------|----------|-----|-------------|-----------------|---------------|
| Lilium Thunb.            | Du 08001, Fushun, Liaoning, China | 108.20±7.57 | 41.55±4.39 | 2.62±0.21 | 424±121 | 1.22±0.20 | Martagon     |
| Lilium Thunb.            | Wang 11001, Shengnongjia, Hubei, China | 113.20±8.69 | 40.14±2.67 | 2.83±0.22 | 676±204 | 1.50±0.19 | Martagon     |
| Lilium Thunb.            | Du 09001, Foping, Shaanxi, China  | 110.97±7.80 | 38.95±2.82 | 2.86±0.22 | 684±198 | 1.49±0.17 | Martagon     |
| Lilium Thunb.            | Du 11001, Chongqing, China       | 111.22±11.17 | 40.76±3.68 | 2.74±0.30 | 675±194 | 1.43±0.21 | Martagon     |
| Lilium Thunb.            | Du 09002, Hanzhong, Shaanxi, China | 111.25±7.94 | 41.23±4.12 | 2.72±0.26 | 489±124 | 1.37±0.25 | Martagon     |
| Lilium Thunb.            | Du 11002, Yichang, Hubei, China  | 109.59±7.84 | 40.08±4.67 | 2.62±0.20 | 658±207 | 1.37±0.18 | Martagon     |
| Lilium Thunb.            | Du 11003, Zigui, Hubei, China    | 109.91±14.7 | 42.88±5.24 | 2.57±0.29 | 795±250 | 1.35±0.20 | Martagon     |
| Lilium Thunb.            | Du 09003, Taihui, Shaanxi, China | 111.91±9.26 | 42.65±4.05 | 2.64±0.23 | 645±193 | 1.49±0.25 | Martagon     |
| Lilium Thunb.            | Wang 11002, Yichang, Hubei, China | 113.72±8.91 | 39.62±3.35 | 2.88±0.28 | 664±226 | 1.35±0.23 | Martagon     |
| Lilium Thunb.            | Du 11004, Yichang, Hubei China   | 112.03±6.39 | 41.74±3.2  | 2.70±0.24 | 815±278 | 1.48±0.21 | Martagon     |
| Lilium Nakai et Kamibayashi | Jia 07001, Changbai Mountains, Jilin, China | 88.34±4.52 | 32.90±2.66 | 2.70±0.23 | 829±197 | 1.46±0.17 | Martagon     |
| Lilium Nakai et Kamibayashi | Du 11005, Fushun, Liaoning, China | 94.78±5.78 | 36.23±2.64 | 2.62±0.17 | 686±363 | 1.49±0.20 | Martagon     |
| Ltsingtaense Gilg        | Du 11006, Tsingtao, Shandong, China | 104.70±6.16 | 44.52±2.23 | 2.36±0.17 | 10.06±2.07 | 1.64±0.35 | Martagon     |
| Lsauricicum Ker-Gawl.    | Du 11007, Fushun, Liaoning, China | 99.21±4.72 | 37.85±4.29 | 2.64±0.24 | 657±264 | 1.54±0.20 | Martagon     |
| Ldavidii Duchartre ex Elwes | Wang 11002, Baoxing, Sichuan, China | 95.50±4.35 | 33.36±2.44 | 2.88±0.23 | 618±215 | 1.28±0.16 | Martagon     |
| Ldavidii Duchartre ex Elwes | Du 11008, Zigui, Hubei, China    | 94.22±5.11 | 34.48±2.82 | 2.75±0.21 | 538±187 | 1.17±0.14 | Martagon     |
| Ldavidii Duchartre ex Elwes | Wang 11003, Chongqing, China    | 85.56±6.34 | 32.45±3.95 | 2.66±0.29 | 567±188 | 1.15±0.16 | Martagon     |
| Ldavidii Duchartre ex Elwes | Du 11009, Lijiang, Yunnan, China | 97.07±5.95 | 33.92±2.15 | 2.87±0.17 | 678±196 | 1.45±0.96 | Martagon     |
| Ldavidii var. willmottiae (E. H. Wilson) Raffill | Jia 07002, Lanzhou, Gansu, China | 95.64±7.13 | 32.75±3.00 | 2.93±0.22 | 527±56 | 1.23±0.21 | Martagon     |
| Ldavidii var. willmottiae (E. H. Wilson) Raffill | Wang 11004, Heqing, Yunnan, China | 88.14±3.82 | 34.43±2.28 | 2.57±0.26 | 579±151 | 1.20±0.14 | Martagon     |
| Lduchartreii Franch.     | Wang 11005, Baoxing, Sichuan, China | 85.07±10.81 | 33.31±3.70 | 2.56±0.23 | 831±279 | 1.30±0.15 | Martagon     |
| L. leichlinii Hook. & var. maximowiczi (Regel) Baker | Du 12001, Beijing Botanical Garden, Beijing, China | 76.86±5.36 | 31.45±3.03 | 2.45±0.18 | 578±255 | 1.21±0.14 | Martagon     |
| L. leichlinii Hook. & var. maximowiczi (Regel) Baker | Du 11010, Fushun, Liaoning, China | 88.75±5.26 | 34.63±2.54 | 2.58±0.23 | 739±231 | 1.29±0.17 | Martagon     |
| L. concolor Salisb. var. pulchellum (Fisch.) Regel | Du 11011, Fushun, Liaoning, China | 71.03±4.66 | 30.56±2.42 | 2.34±0.21 | 571±153 | 1.21±0.36 | Concolor     |
| L. concolor Salisb. var. pulchellum (Fisch.) Regel | Du 11012, Fushun, Liaoning, China | 70.51±5.13 | 30.59±2.44 | 2.31±0.11 | 350±179 | 1.50±0.20 | Concolor     |
| L. pulnim DC.            | Jia 09001, Tongliang, Inner Mongolia, China | 87.39±7.00 | 32.66±1.82 | 2.67±0.12 | 496±190 | 1.35±0.22 | Martagon     |
| Litaluenese Franch.      | Jia 09002, Chongqing, China      | 79.54±8.11 | 29.91±2.39 | 2.59±0.26 | 612±203 | 1.15±0.18 | Martagon     |
| Litaluenese Franch.      | Du 12002, Shangri-la, Yunnan, China | 69.85±3.31 | 29.49±3.42 | 2.40±0.27 | 357±11 | 1.07±0.09 | Martagon     |
| Lwardii Stapf ex Stearn | Du 11013, Bomu, Tibet        | 87.77±5.97 | 35.26±1.97 | 2.49±0.18 | 748±220 | 1.46±0.15 | Martagon     |
| Samples                      | Voucher                      | P (μm) * | E (μm) # | P/E | Lumina (μm) | Muri width (μm) | Pollen type** |
|------------------------------|------------------------------|----------|----------|-----|-------------|-----------------|---------------|
| L. bakerianum Coll. et Hemsli| Du 12003, Lijiang, Yunnan, China | 78.40±4.88 | 79.71±15.81 | 1.03±0.19 | 5.84±3.42 | 1.46±0.35 | Concolor |
| L. brownii F. E. Brown ex Miellez | Wang 11006, Shennongjia, Hubei, China | 120.13±3.65 | 53.16±6.50 | 2.29±0.29 | 12.29±4.49 | 1.69±0.18 | Martagon |
| L. brownii F. E. Brown ex Miellez | Du 09004, Foping, Shaanxi, China | 103.18±9.16 | 46.11±4.48 | 2.25±0.20 | 7.95±2.74 | 1.68±0.24 | Martagon |
| L. brownii F. E. Brown ex Miellez | Du 11014, Yichang, Hubei, China | 109.77±4.24 | 50.91±5.39 | 2.18±0.23 | 10.44±4.37 | 1.86±0.28 | Martagon |
| L. henryi | Du 11015, Zigui, Hubei, China | 92.67±4.65 | 35.98±2.70 | 2.59±0.18 | 4.36±2.40 | 1.11±0.15 | Martagon |
| L. henryi | Du 12004, Beijing botanical garden, Beijing, China | 96.47±5.35 | 34.86±2.62 | 2.78±0.20 | 6.46±2.03 | 1.37±0.31 | Martagon |
| L. rosthornii | Jia 10001, Chongqing, China | 98.01±5.87 | 35.20±2.38 | 2.79±0.17 | 5.08±1.60 | 1.06±0.11 | Martagon |
| L. rosthornii | Du 12005, Yuanling, Hunan, China | 92.31±6.88 | 32.88±2.28 | 2.81±0.18 | 6.40±2.20 | 1.20±0.26 | Martagon |
| L. regale Wilson | Du 11015, Maoxian, Sichuan, China | 100.68±6.00 | 41.09±3.76 | 2.47±0.24 | 9.32±2.85 | 1.57±0.29 | Callose |
| L. regale Wilson | Wang 11007, Lixian, Sichuan, China | 93.55±4.40 | 42.62±2.97 | 2.20±0.15 | 9.94±3.48 | 2.22±0.46 | Callose |
| L. regale Wilson | Du 11016, Wenchuan, Sichuan, China | 95.78±5.15 | 39.95±2.88 | 2.41±0.21 | 10.26±3.55 | 1.52±0.47 | Callose |
| L. regale Wilson | Du 12006, Beijing botanical garden, Beijing, China | 99.33±5.16 | 40.16±3.92 | 2.49±0.22 | 8.93±2.91 | 1.79±0.25 | Callose |
| L. sargentiae Wilson | Jia 11001, Chongqing, China | 91.33±4.65 | 33.03±2.50 | 2.78±0.25 | 6.29±1.83 | 1.08±0.21 | Callose |
| L. sargentiae Wilson | Du 11017, Baokeng, Sichuan, China | 95.11±7.01 | 35.96±2.25 | 2.65±0.18 | 6.49±1.97 | 1.16±0.16 | Callose |
| L. sargentiae Wilson | Du 11018, Baokeng, Sichuan, China | 89.83±5.75 | 33.60±2.36 | 2.68±0.15 | 6.50±1.95 | 1.35±0.14 | Callose |
| L. sargentiae Wilson | Wang 11008, Chongqing, China | 92.10±4.90 | 35.67±2.92 | 2.60±0.26 | 6.52±1.95 | 1.66±0.23 | Callose |
| L. sargentiae Wilson | Wang 11009, Chongqing, China | 89.08±6.62 | 33.28±2.33 | 2.68±0.20 | 7.34±2.00 | 1.33±0.19 | Callose |
| L. leucanthum Baker | Du 11019, Zigui, Hubei, China | 98.82±9.59 | 36.55±3.72 | 2.71±9.16 | 6.80±2.05 | 1.46±0.18 | Callose |
| L. sulphureum Baker apud Hook. f. | Du 12007, Beijing botanical garden, Beijing, China | 87.56±4.58 | 38.69±3.58 | 2.28±0.23 | 6.40±2.01 | 1.50±0.30 | Callose |
| L. formosanum Wallace | Du 12008, Beijing botanical garden, Beijing, China | 122.30±7.64 | 49.89±4.22 | 2.46±0.20 | 12.63±3.71 | 1.22±0.39 | Formosanum (this study) |
| L. cernuum Kom. | Zhang et al.(2006) | 108.9 | 44.9 | 2.43 | 4.99 | 2.1 | Martagon |
| L. amabile Palib. | Zhang et al.(2006) | 106.2 | 41.8 | 2.54 | 7.55 | 1.93 | Martagon |
| L. leucanthum var. centifolium (Stapf ex Elwes) Stearn | Zhang et al.(2010) | 74.3 | 35.1 | 2.12 | 3.3 | 2.4 | Martagon |
| L. henrici Franch. | Liang and Zhang (1985) | 70.5 | 47.0 | | | | Concolor |
| L. jephthophorum(Bur.et Franch.) Franch | Liang and Zhang (1985) | 70.5 | 65.8 | | | | Martagon |
| L. nanum Klotz. Et Garcke | Liang and Zhang (1985) | 84.6 | 61.1 | | | | Martagon |
| L. nanum Klotz. Et Garcke var. brevistylum S.L. Liang and Zhang (1985) | Liang | 58.8 | 49.4 | | | | Martagon |
| L. sempervivoidum Lev. | Liang and Zhang (1984) | 103.4 | 117.5 | | | | Martagon |
### Table 1. Cont.

| Samples              | Voucher                        | $P$ (μm) * | $E$ (μm) # | P/E | Lumina (μm) | Muri width (μm) | Pollen type** |
|----------------------|--------------------------------|------------|------------|-----|------------|-----------------|--------------|
| L. amoenum Wilson ex Sealy | Liang and Zhang (1984)       | 112.8      | 122.2      |     |            |                 | Martagon     |
| L. speciosum Thunb. var. gloriosoides Baker | Wu et al. (2007)            | 59.5       | 21.4       | 2.78 | 4.68       | 1.03            | Martagon     |
| L. martagon L.         | Halbritter (1993)            |            |            |     |            |                 | Martagon     |
| L. basniacum (Beck)    | Muratović et al. (2010)      |            |            |     |            |                 | Martagon     |
| L. camilicium Bernh. ex W.D.J. Koch | Muratović et al. (2010)     |            |            |     |            |                 | Martagon     |
| L. canadense           | Kosenko (1999)               | 74.8–76.8  | 51.8–55.8  |     |            |                 | Martagon     |
| L. columbianum         | Kosenko (1999)               | 71.0–76.8  | 44.1–46.0  |     |            |                 | Martagon     |
| L. kesselringianum     | Kosenko (1999)               | 101.8–107.5| 53.7–59.5  |     |            |                 | Martagon     |

Note: * Polar axis; # Equatorial axis; ** Following the nomenclature of Baranova (1985).

### Table 2. Variance analysis of pollen morphology traits among species from different provenances.

| Traits | Species                                      | L. lancifolium | L. regale | L. davidii | L. sargentiae | L. brownii | L. davidii var. wilflortiae | L. distichum | L. henryi | L. concolor var. pulchelum | L. rosthornii | L. leichtlinii var. maximowiczii |
|--------|----------------------------------------------|----------------|-----------|------------|---------------|-------------|-------------------------------|--------------|-----------|-------------------------------|---------------|---------------------------------|
| P      |                                              | 0.551          | 9.278**   | 14.763**   | 3.613**       | 10.40**     | 6.705*                        | 20.757**     | 6.786*    | 0.086                         | 7.964**       | 31.007**                         |
| E      |                                              | 2.426*         | 2.830°    | 1.526      | 6.811**       | 5.539**     | 2.068                         | 23.439**     | 2.061     | 0.001                         | 10.597**      | 6.793*                           |
| P/E    |                                              | 3.764**        | 9.996**   | 3.347*     | 1.8           | 0.61        | 14.288**                      | 2.165        | 12.115**  | 0.186                         | 0.164         | 2.742                           |
| Lumina |                                              | 10.289**       | 0.837     | 5.638**    | 1.502         | 14.393**    | 2.994                         | 5.163*       | 41.913**  | 66.946**                      | 16.479**      | 17.303**                         |
| Muri   |                                              | 3.62**         | 51.346**  | 36.556**   | 65.017**      | 14.983      | 0.169                         | 0.426        | 0.017     | 4.616*                        | 21.425**      | 6.474*                           |

Note: * represents the significant difference ($p<0.05$), and ** represents the extremely significant difference ($p<0.01$).
Results

Numerical results of the palynological study are summarized in Table 1. According to the Lilium classification by Comber [8], Nishikawa et al. [18,19] and Du et al. (2013) [16], palynological results for each section are as follows:

Martagon

Representatives of Martagon (Figure 1A–F) have pollen size ranging from L. distichum Nakai et Kamibayashi (91.56 × 94.57 µm, P/E of 2.66) to L. tsingtauense (111.20 × 91.66 µm, P/E of 2.72); L. davidi Duchartre ex Eltes (93.09 × 93.55 µm, P/E of 2.79); L. davidi var. willmotiae (E. H. Wilson) Raffill (91.89 × 93.59, P/E of 2.75); L. davidi Duchartre ex Eltes (85.07 × 83.31, P/E of 2.50); L. leucanthum Hook. f. var. maximowiczii (Regel) Baker (82.81 × 83.04, P/E of 2.52); L. pumilum DC (87.39 × 92.66, P/E of 2.67); L. concolor Salisb. var. pulchellum (Fisch.) Regel (70.77 × 30.58, P/E of 2.33); L. wardii Stapf ex Stearn (70.77 × 35.26, P/E of 2.49); and L. talense Franck (74.70 × 29.70, P/E of 2.50) (Table 1).

SEM of pollen from this section showed differences in exine sculpture. L. concolor var. pulchellum clearly shows Concolor type pollen (Figure 4E–H), whereas pollen from other Sinomartagon species show rectangular columellae that correspond to Martagon type.

Sinomartagon

Triploid L. lancifolium pollen is relatively larger than the other diploid species in section Sinomartagon. The shape can be ellipsoidal to long-ellipsoidal. Species belonging to Sinomartagon (Figure 1M–P; 2A–P; 3A–P; 4A–P) show a decreasing tendency in size with regard to pollen features from subsect. 5a to subsect. 5c as follows: L. lancifolium Thunb (111.20 × 94.16 µm, P/E of 2.72); L. davidi Duchartre ex Eltes (93.09 × 93.55 µm, P/E of 2.79); L. davidi var. willmotiae (E. H. Wilson) Raffill (91.89 × 93.59, P/E of 2.75); L. davidi Duchartre ex Eltes (85.07 × 83.31, P/E of 2.50); L. leucanthum Hook. f. var. maximowiczii (Regel) Baker (82.81 × 83.04, P/E of 2.52); L. pumilum DC (87.39 × 92.66, P/E of 2.67); L. concolor Salisb. var. pulchellum (Fisch.) Regel (70.77 × 30.58, P/E of 2.33); L. wardii Stapf ex Stearn (70.77 × 35.26, P/E of 2.49); and L. talense Franck (74.70 × 29.70, P/E of 2.50) (Table 1).

SEM of pollen from this section showed differences in exine sculpture. L. concolor var. pulchellum clearly shows Concolor type pollen (Figure 4E–H), whereas pollen from other Sinomartagon species show rectangular columellae that correspond to Martagon type.

Leucolirion

Representatives of Leucolirion (Figure 1G–L; 5C–P; 6A–P; 7A–B) have pollen sizes ranging from 87.56 × 36.69, P/E of 2.28 for L. sulphureum Baker apud Hook. f. to 120.13 × 53.16, P/E of 2.29 for L. brownii from Shennongjia in the Hubei Province. The exine is ornamented with reticulation, and the sculpture is ellipsoidal to long-ellipsoidal. Pollen from L. brownii, L. henryi, and L. rothmani correspond to the Martagon type (Figure 1G–L; 5C–J). The pollen exine of L. regale Wilson, L. leucanthum (Baker) Baker, L. sargentiae Wilson and L. sulphureum show rounded columellae, corresponding to the Callose type (Figure 5M–P; 6A–P). Pollen of L. formosanum Wallace does not correspond to any of the pollen types proposed by Banarova (1985), and the exine surface is reticulate with solid muri or irregular-rugulate (Figure 7A–B). We suggest creating a new category of Formosanum type pollen.

Lophophorum

The division of Lophophorum remains controversial. Due to resource constraints, we selected L. bakerianum as the only representative. In a preliminary study, L. bakerianum was placed in the subsect. Lophophorum III [16]. Pollen tetrads were found in L. bakerianum (Figure 5A–B), observed as blunt quadrangles in a polar view with a pollen size of 78.40 × 79.71 µm, P/E of 1.03. The exine ornamentation was reticulate with muri formed by separated rounded columellae, corresponding to the Callose type. This is the first study to report that pollen grains of L. bakerianum are tetrads.

Discussion

Pollen Morphology and Phylogenetic Implications Based on ITS Sequences

Within the genus Lilium, several diversified pollen exine sculptures were observed. Pollen sculpture patterns appear to reflect phylogenetic relationships and are useful for species or subsection delimitations within a section.

The phylogenetic tree was resolved into six groups based on ITS sequences (Figure 8). Group I is comprised of three clades. Present and previous molecular analyses [18,19] and cytology studies [21,43–44] indicated that sect. Sinomartagon is complicated and polyphyletic, as supported by the pollen data (Figure 8). Species of sect. Sinomartagon (sect. 5a&5b) and L. formosanum and L. brownii (sect. Leucolirion 6b) formed a clade with strong support ([ML] BS = 91, [MP] BS = 77). Sect. Sinomartagon species show two pollen types, Martagon and Concolor. Differences in the shape and arrangement of the types of columellae could have implications on species delimitation. L. concolor var. pulchellum clearly shows Concolor type pollen (Figure 4G–J), and the remainder show Martagon type pollen. Meanwhile, three subtypes were showed within Martagon type pollen (Figure 6A, B, C). Exine sculptures of L. davidi and L. davidi var. willmotiae showed spherical but dorsally and ventrally compressed, and loosely arranged columellae with protuberant and enlarged columellae at the intersection of the muri (Figure 3B–N; 99B). Muri of L. leucanthum var. maximowiczii have regular long-rectangular columellae (Figure 3O–P; 9C). In addition, L. formosanum and L. brownii (Leucolirion 6b) formed a strongly supported clade ([ML] BS = 87, [MP] BS = 86) (Figure 8). In the cytological study, both L. formosanum and L. brownii lacked intercalary satellites on the first two pairs of chromosomes [43,46]. However, the pollen of L. formosanum corresponds to the new Formosanum type, while L. brownii clearly shows Martagon type pollen.

In our analysis, L. talense and L. wardii ([ML] BS = 90, [MP] BS = 85) formed a clade with L. amoenum, L. sempervivoideum and L. bakerianum ([ML] BS = 79, [MP] BS = 84) that was moderately supported ([ML] BS = 56, [MP] BS < 50) (Figure 8). In cytological studies, the L. talense karyotype resembles that of L. wardii [21,43]. Morphologically, L. talense and L. wardii showed a close relationship with each other, with a black line in the central groove of the tepals [2]. Pollen evidence from our study supports this result, as the pollen morphology of the two species is generally similar Martagon type (Figure 4K–P). Morphologically, L. amoenum, L. sempervivoideum and L. bakerianum show nodding or horizontal campanulate flowers, nectaries of inner tepals neither papillose nor with fimbriate projections, tepals without blotch at the base adaxially, and a papillose stem [2]. The L. bakerianum var. delavayi karyotype resembles that of L. sempervivoideum [43]. Pollen tetrads are present in L. amoenum, L. sempervivoideum and L. bakerianum (Figure 5A–B). The similarity in pollen morphology also suggests a close relationship with this clade.

The Martagon clade was monophyletic with strong support ([ML] BS = 90,[MP] BS = 96), which is consistent with previous studies by Gao et al. [13] and Nishikawa et al. [18,19]. Morphologically, they all show whorled leaves. Although the Martagon clade shows Martagon type pollen, there are minor differences among species in the Martagon clade in the protuberant
| Samples                  | Voucher              | Latitude (°) | Longitude (°) | Altitude (m) | Annual average temperature (°C) | Annual average sunshine (h) | Yearly precipitation (mm) |
|-------------------------|----------------------|--------------|---------------|--------------|---------------------------------|-----------------------------|---------------------------|
| *L. lancifolium* Thunb. | Du 08001, Fushun, Liaoning, China | 42.09        | 124.43        | 450          | 7                               | 780                         | 2300                      |
| *L. lancifolium* Thunb. | Wang 11001, Shengnongjia, Hubei, China | 39.59        | 116.12        | 1200         | 12                              | 1600                       | 1858                      |
| *L. lancifolium* Thunb. | Du 09001, Foping, Shaanxi, China | 31.00        | 110.58        | 721          | 13.6                            | 1500                       | 1669                      |
| *L. lancifolium* Thunb. | Du 11001, Chongqing, China | 31.05        | 110.54        | 1510         | 13.6                            | 1500                       | 1669                      |
| *L. lancifolium* Thunb. | Du 09003, Taibai, Shaanxi, China | 31.01        | 110.57        | 1018         | 13.6                            | 1500                       | 1669                      |
| *L. lancifolium* Thunb. | Du 11002, Yichang, Hubei, China | 31.02        | 110.57        | 1110         | 13.6                            | 1500                       | 1669                      |
| *L. lancifolium* Thunb. | Du 11004, Yichang, Hubei China | 31.02        | 110.57        | 1110         | 13.6                            | 1500                       | 1669                      |
| *L. distichum* Nakai et Kamibayashi | Jia 07001, Changbai Mountains, Jilin, China | 42.07        | 124.45        | 450          | 7                               | 780                         | 2300                      |
| *L. distichum* Nakai et Kamibayashi | Du 11005, Fushun, Liaoning, China | 41.47        | 124.44        | 475          | 7                               | 780                         | 2300                      |
| *L. tsingtauense* Gilg | Du 11006, Tsingtao, Shandong, China | 36.09        | 120.37        | 426          | 12.1                            | 775.6                       | 2503                      |
| *L. dauricum* Ker-Gawl. | Du 11007, Fushun, Liaoning, China | 42.06        | 124.48        | 365          | 7                               | 780                         | 2300                      |
| *L. davidii* Duchartre ex Elwes | Wang 11002, Baoding, Sichuan, China | 31.05        | 110.53        | 1557         | 13.6                            | 1500                       | 1669                      |
| *L. davidii* Duchartre ex Elwes | Du 11008, Zigu, Hubei, China | 31.05        | 110.53        | 1557         | 13.6                            | 1500                       | 1669                      |
| *L. davidii* Duchartre ex Elwes | Wang 11003, Chongqing, China | 29.03        | 107.12        | 883          | 17                              | 1395                       | 1079                      |
| *L. davidii* Duchartre ex Elwes | Du 11009, Lijiang, Yunnan, China | 41.34        | 122.32        | 2533         | 16.5                            | 1000                       | 2400                      |
| *L. davidii* var. willmottiae (E. H. Wilson Raffill) | Jia 07002, Lanzhou, Gansu, China | 26.19        | 100.09        | 2408         | 15.5                            | 1050                       | 2253                      |
| *L. davidii* var. willmottiae (E. H. Wilson Raffill) | Wang 11004, Heqing, Yunnan, China | 30.50        | 102.43        | 2859         | 14.1                            | 994                        | 789                       |
| *L. leichtlinii* Hook.f. var. maximowiczii (Regel) Baker | Du 12001, Beijing Botanical Garden, Beijing, China | 41.46        | 124.42        | 324          | 7                               | 780                        | 2300                      |
| *L. leichtlinii* Hook.f. var. maximowiczii (Regel) Baker | Du 11010, Fushun, Liaoning, China | 41.46        | 124.43        | 388          | 7                               | 780                        | 2300                      |
| *L. concolor* Salisb. var. pulchellum (Fisch.) Regel | Du 11011, Fushun, Liaoning, China | 42.06        | 124.47        | 330          | 7                               | 780                        | 2300                      |
| *L. concolor* Salisb. var. pulchellum (Fisch.) Regel | Du 11012, Fushun, Liaoning, China | 41.35        | 124.09        | 324          | 7                               | 780                        | 2300                      |
| *L. pumilum* DC. | Jia 09001, Tongliao, Inner Mongolia, China | 33.24        | 108.76        | 813          | 10                              | 938                        | 1726                      |
| *L. taliense* Franch. | Jia 09002, Chongqing, China | 29.03        | 107.12        | 1265         | 17                              | 1395                       | 1079                      |
| *L. taliense* Franch. | Du 12002, Shangri-la Yunnan, China | 27.52        | 99.49        | 3191         | 11                              | 650                        | 2000                      |
| *L. wardii* Stapf ex Stearn | Du 11013, Bomi, Tibet | 30.06        | 95.04        | 2053         | 8.5                             | 977                        | 1563                      |
| *L. bakerianum* Coll. et Hemsl | Du 12003, Lijiang, Yunnan, China | 26.28        | 100.43        | 1848         | 13.6                            | 600                        | 2403                      |
### Table 3. Cont.

| Samples | Voucher                                      | Latitude (°) | Longitude (°) | Altitude (m) | Annual average temperature (°C) | Annual average sunshine (h) | Yearly precipitation (mm) |
|---------|----------------------------------------------|--------------|---------------|--------------|---------------------------------|-----------------------------|---------------------------|
| L. brownii F. E. Brown ex Miellez Wang 11006, Shennongjia, Hubei, China | 39.59         | 116.12        | 1200          | 12                 | 1600                          | 1858                       |
| L. brownii F. E. Brown ex Miellez Du 09004, Foping, Shaanxi, China    | 33.24         | 108.76        | 813           | 10                 | 938                           | 1726                       |
| L. brownii F. E. Brown ex Miellez Du 11014, Yichang, Hubei, China     | 31.01         | 110.57        | 1004          | 13.6               | 600                           | 2403                       |
| L. henryi Bake Du 11015, Zigui, Hubei, China                          | 28.49         | 110.23        | 875           | 13.6               | 600                           | 2403                       |
| L. henryi Bake Du 12004, Beijing botanical garden, Beijing, China      | 27.54         | 99.57         | 396           | 16.6               | 1437                          | 1200                       |
| L. rosthornii Diels Jia 10001, Chongqing, China                      | 31.42         | 103.51        | 1504          | 11.2               | 491                           |                            |
| L. regale Wilson Du 11015, Maoxian, Sichuan, China                   | 31.26         | 103.09        | 1910          | 9                  | 800                           |                            |
| L. regale Wilson Du 11016, Wenchuan, Sichuan, China                  | 31.27         | 103.33        | 1409          | 14                 | 920                           |                            |
| L. regale Wilson Du 12006, Beijing botanical garden, Beijing, China   | 29.03         | 107.12        | 918           | 17                 | 1396                          | 1079                       |
| L. sargentiae Wilson Jia 11001, Chongqing, China                     | 30.36         | 102.84        | 1015          | 14.1               | 994                           | 789                        |
| L. sargentiae Wilson Du 11017, Baoxing, Sichuan, China               | 29.04         | 107.16        | 787           | 17                 | 1396                          | 1079                       |
| L. sargentiae Wilson Wang 11009, Chongqing, China                    | 31.05         | 110.54        | 1525          | 13.6               | 1500                          | 1669                       |
| L. sulphureum Baker apud Hook, f. Du 12007, Beijing botanical garden, Beijing, China | 31.05         | 110.54        | 1525          | 13.6               | 1500                          | 1669                       |
| L. formosanum Wallace Du 12008, Beijing botanical garden, Beijing, China | 31.05         | 110.54        | 1525          | 13.6               | 1500                          | 1669                       |

Note: Climate parameters were obtained from the local meteorological bureau.

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and enlarged colulemata at the intersection of the muri in *L. tsingtauense* (Figure 1E–F, 8K).

Group II was weakly supported ([ML] BS<50, [MP] BS<50) and consisted of two Lophophorum clades. Lophophorum clade I consisted of *L. lophophorum* and *L. nanum* ([ML] BS = 77 and [MP] BS = 71). In palynological research, *L. lophophorum* and *L. nanum* are monocotopole or 2–3 porate and show similar pollen morphologies (Figure 8L) [24]. Representatives of *L. henricii* in subsect. Lophophorum II show Concolor type pollen with much smaller rectangular colulemata between two separate irregular round colulemata compared with *L. concolor* var. *pulchellum* (Figure 8M) [24]. Morphologically, representatives of the Lophophorum clade I show nectaries on the inner tepals with fimbriate/cristate projections on both surfaces, while representatives of subsect. Lophophorum II do not have this feature. The distinct pollen morphology of the species suggests a distinct relationship to support the molecular data [2].

In Group III, the Pseudolirium clade was resolved with strong support ([ML] BS = 77, [MP] BS = 86). Pollen morphologies from representatives of sect. Pseudolirium were similar, with loosely arranged colulemata and protuberant and enlarged colulemata at the intersection of the muri (Figure 8N) [35].

Group IV, consisting of *L. kesselringianum*, *L. bosniacum* and *L. carniolicum*, was robustly supported ([ML] BS = 97, [MP] BS = 97). The three representatives of European species (sect. Liriopytus) showed Martagon type pollen with densely arranged columellae forming the muri (Figure 8O) [25].

Group V was only comprised of *L. duchartrei* (Sinomartagon clade I) (Figure 8). Based on morphology, Comber classified it in subsection Sinomartagon 5a. However, in our ITS analyses, *L. duchartrei* showed a distant relationship with subsect. 5a species as well as other sect. Sinomartagon species (Figure 8), which is in accordance with previous studies [13,18–19]. *L. duchartrei* (Sinomartagon clade I) karyotypes have been shown to be dissimilar to subsect. 5a Sinomartagon clade I species [21,43]. *L. duchartrei* pollen resembles that of *L. taliense* and *L. varidi* in exine sculpture (Figure 4C–D; K–P). Given that the phylogenetic position of *L. duchartrei* is uncertain, classification as a subsection of sect. Sinomartagon may be reasonable.

In Group VI, *L. speciosum* var. *gloriosoides* in sect. Archelirion formed a weakly supported group consisting of representatives of Leucolirion 6a ([ML] BS<50 and [MP] BS<50). Leucolirion 6a comprised two subclades ([ML] BS = 74 and [MP] BS = 75). *L. speciosum* var. *gloriosoides* showed a distinct relationship with *L. henryi* and *L. rosthornii* in the ITS tree, although they all have Martagon-type pollen (Figure 5). In addition, *L. henryi* and *L. rosthornii*, which have orange reflexed flowers with prominent papillae and pubescent nectaries, were resolved with strong support ([ML] BS = 98, [MP] BS = 100). Species of *L. leucanthemum*, *L. sargentiae*, *L. sulphurum* and *L. regale* formed a clade with strong support ([ML] BS = 95, [MP] BS = 95). Pollen morphology from our study supports this result, as the pollen morphology of *L. henryi* resembles *L. rosthornii* as Martagon type (Figure 5C–H), while *L. leucanthemum*, *L. sargentiae*, *L. sulphurum* and *L. regale* have all generally similar Callose-type pollen (Figure 5K–L; 6A–P).

Within *Lilium*, we noted that one section may show two or more different pollen types (Figure 6). For instance, sect. Sinomartagon shows two exine sculpture pollen types, Martagon and Concolor, and the former contains three different subtypes. No unique and uniform characters can be used to clearly distinguish sections or some subsections due to shared or overlapping characters.

### Trends in Exine Sculpture Evolution and Factors Implicated in Pollen Evolutionary Trends

Changes in pollen morphology occur in response to selective pressure. Pollen grains should be considered a functional unit, with the exine structure as a compromise between the following four main functions [32]: protective, harmomegathic, reservoir and clustering. These functions are in response to physical components of the environment, such as water, nutrients, temperatures and growing season.

The genus *Lilium* is distributed in East Asia, the Himalayas and Hengduan Mountains, Europe and North America [1,2]. Patterson and Givnish [4] concluded that *Lilium* evolved in the Himalayas and then dispersed into Eurasia and North America, although intercontinental dispersal details are not clear [4]. In the karyotype study, the relatively primitive group (sect. Lophophorum (Bur. et Franch.) Wang et Tang) have no intercalary satellites on the first two pairs of chromosomes. As expected, the genus *Notholirion* which is commonly considered to be closely related to and more ancient than *Lilium* have also been found to lack intercalary satellites [4,2,43,45,46], these species are distributed mainly in the Himalayas, which makes this region more likely as the origin of the genus *Lilium*. Besides, Stewart [43] pointed out that secondary constrictions correlated with chromatin distribution as well as with geographic distribution. In his study, most East Asian and Himalayan species were found to have intercalary satellites on the first two pairs of submedian centromeres, while all North American species and European species lack such feature [21,25,43,45]. Hence, the situation among the Himalayan and Hengduan Mountains species appears more complicated since they seem to have both types, even within the same section indicating that the Hengduan Mountains are more likely as the differentiation centre of the genus [43,45,47]. Pollen evidence from our study supports this hypothesis, as the pollen morphology of the species from the Himalayas and Hengduan Mountains contain all of the pollen types in the genus *Lilium*. It should be emphasized

### Table 4. Correlation coefficient between the main characteristic of pollen morphology and the geographical and climate factors of provenances.

| Traits | Latitude (°) | Longitude (°) | Altitude (m) | Annual average temperature (°C) | Annual average precipitation (mm) | Annual average sunshine (h) |
|--------|--------------|---------------|--------------|----------------------------------|-----------------------------------|-----------------------------|
| P      | 0.046        | 0.082         | -0.013       | 0.172                            | 0.413*                            | -0.013                      |
| E      | -0.105       | -0.103        | 0.041        | 0.014                            | -0.129                            | 0.249                       |
| P/E    | 0.133        | 0.178         | -0.137       | 0.160                            | 0.442**                           | -0.251                      |
| Lumina | 0.010        | -0.073        | 0.039        | 0.073                            | 0.024                             | -0.091                      |
| Muri   | 0.126        | 0.048         | -0.035       | -0.199                           | -0.189                            | 0.134                       |

Note: * represents the significant difference (p<0.05), and ** represents the extremely significant difference (p<0.01).

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that the Formosanum type of pollen can be found in the genus *Nomocharis*, which is restricted to the Himalayas and Hengduan Mountains and it has been suggested that it be included in *Lilium* [13–16]. In contrast, the other regions possess a relatively singular pollen type, which led us to investigate *Lilium* pollen-type evolution.

Based on a pollen study of 30 genera in Liliaceae, including 69 species, using light microscopy (LM), Nair and Sharma [46]
Figure 2. SEM photographs of pollen grains of *Lilium*. Figs A, B, *L. lancifolium* (Du 09001). Figs C, D, *L. lancifolium* (Du 11001). Figs E, F, *L. lancifolium* (Du 09002). Figs G, H, *L. lancifolium* (Du 11002). Figs I, J, *L. lancifolium* (Du 11003). Figs K, L, *L. lancifolium* (Du 09003). Figs M, N, *L. lancifolium* (Wang 11002). Figs O, P, *L. lancifolium* (Du 11004). Scale bars: 10 μm.
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Figure 3. SEM photographs of pollen grains of *Lilium*. Figs A, B, *L. dauricum*. Figs C, D, *L. davidii* (Wang 11002). Figs E, F, *L. davidii* (Du 11008). Figs G, H, *L. davidii* (Wang 11003). Figs I, J, *L. davidii* (Du 11009). Figs K, L, *L. davidii* var. *willmottiae* (Jia 07002). Figs M, N, *L. davidii* var. *willmottiae* (Wang 11004). Figs O, P, *L. leichtlinii* var. *maximowiczii* (Du 12001). Scale bars: 10 μm.

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Figure 4. SEM photographs of pollen grains of *Lilium*. Figs A, B, *L. leichtlinii* var. *maximowiczii* (Du 11010). Figs C, D, *L. duchartrei*. Figs E, F, *L. pumilum*. Figs G, H, *L. concolor* var. *pulchellum* (Du 11011). Figs I, J, *L. concolor* var. *pulchellum* (Du 11012). Figs K, L, *L. taliense* (Jia 09002). Figs M, N, *L. taliense* (Du 12002). Figs O, P, *L. wardii*. Scale bars: 10 µm.

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Figure 5. SEM photographs of pollen grains of *Lilium*. Figs A, B, *L.* bakerianum. Figs C, D, *L.* henryi (Du 11015). Figs E, F, *L.* henryi (Du 12004). Figs G, H, *L.* rosthornii (Jia 10001). Figs I, J, *L.* rosthornii (Du 12005). Figs K, L, *L.* regale (Du 11015). Figs M, N, *L.* regale (Wang 11007). Figs O, P, *L.* regale (Du 11016). Scale bars: 10 μm.
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proposed the scheme of pollen exine sculpture evolution (Liliaceae) as follows: ornate → reticulate → reticulate → ring-shaped reticulate → scattered reticulate → exineless ornamentation. Regarding pollen morphology of the genus *Nomocharis* and *Lilium* under LM and SEM, Liang and Zhang [24] suggested the following evolution trend of pollen exine sculpture in the genus...
Lilium: ornate → reticulate, which is consistent with the hypothesis of Nair and Sharma [46]. In addition, reticulate was divided into three exine sculptures to form three Lilium pollen types, including Martagon, Callose and Concolor. These results led us to propose the following hypothesis of pollen evolution in exine sculptures within Lilium. Martagon type → Callose type → Concolor type → Formosanum type. This also shows a reduction and simplification tendency.

In monocots, reduction and simplification of the exine structure is even more extreme than in dicots and culminate in certain evolutionary orders [48]. Zavada proposed major evolutionary trends of wall structure types in monocots as follows: primitive tectate-columellate (perforate or imperforate) wall structures give rise to monocotyledonous atectate or granular walls and eventual extreme reduction of the exine, which may be completely absent [48]. All morphological characteristics are the consequence of interactions between phylogenetic and environmental constraints [48]. There is no doubt that environmental constraints act on patterns of differentiation in Lilium [49].

In addition to environmental factors, are there other factors such as pollinator affecting pollen differentiation? Numerous recent findings on the intricate flower pollinator networks suggest that differentiation of flora structures and functions in plants are not only determined by environmental factors, but also influenced by pollinators. Nair and Sharma [46] pointed out that differentiation of flora structures and functions in plants are insect-pollinated. Brantjes and Bos's [54] report that both diurnal and nocturnal hawkmoths visit L. martagon. Skinner [55] observed pollinators of 13 American species of Lilium and recognized five types of pollination: butterfly pollination (L. humboldtii, L. kelleyanum and L. kelloggii), butterfly and hummingbird pollination (L. wigginsi, L. pardinum and L. pardinum ssp. vollmeri), hummingbird pollination (L. columbianum, L. occidentale and L. bolanderi), hummingbird and bumblebee pollination (L. parvum and L. maritimum) and hawkmoth pollination (L. washingtonianum and L. parryi). Additional studies reported various flower visitors, including fritillaries to L. concolor var. pulchellum [56], swallowtails to L. dauricum [56], nocturnal hawkmoths to L. formosanum [57] and L. japonicum [58], and hawkmoths and swallowtails to L. auratum [56,59]. Nevertheless, wild Lilium species are self-compatible. Autogamy might have ensured reproductive success for the species in the environments where pollinators are comparatively rare [60] and the growing season short as well as where other conditions are selective. Pollen of L. martagon and L. dauricum with different pollinators are Martagon type (Table 1) [54,56]. Thus, for Lilium, the pollinators have little effect on changes of pollen morphology and size. Moreover, in Sonneratiaceae, the genus Duabanga has much smaller pollen grains than Sonneratia while both genera are bat-pollinated, hence absence of correlation with pollinator [32]. However, how floral traits as well as pollen grains of lilies are adapted to various pollinators remains to be studied in more detail.

As mentioned, environmental factors constitute a selective pressure that may produce changes in the pollen structure. The number and arrangement of the types of columellae could influence exine flexibility. Exines with loose arrangement of round and size. Moreover, in Sonneratiaceae, the genus Duabanga (Callose, Concolor) are relatively more flexible compared to exines with tight, rectangular columellae (Martagon). This exine structure corresponds to harmomegathic function and the ability to absorb bending stresses that may occur during desiccation [32].

**Relationship between Pollen Size and Conditions in the Genus Lilium**

Species at high elevations with relatively extreme conditions in the Himalayas and Hengduan Mountains have a relatively small pollen size (Table 1). Plants growing at high elevations with extreme conditions often have reduced morphological features [61,62], and pollen size also appears to show this tendency [33,63]. Furthermore, species native to Northeast China, such as L. leichlinii var. maxoniiwiczii, L. concolor var. pulchellum and L. dauricum, also have a relatively small pollen size (Table 1). A long winter and short growing season forces rapid completion of plant growth cycles. Hence, small pollen size also appears to be more efficient in tolerating relatively extreme conditions. In contrast, L. brownii and L. formosanum have relatively bigger pollen grains, likely because they occur in an environment suitable for slow development that allow pollen grains to grow to a larger size, as discussed by Muller [32]. Therefore, pollen size could be related to the selective pressure to adapt to environmental conditions. In
Figure 8. Mapping pollen exine sculpture characters on the ML tree of *Lilium* based on nuclear internal transcribed spacer (ITS) sequence data (adapted from Du et al. unpublished data). Values along branches represent bootstrap (BS) of ML and MP, respectively. A, B, C, D, Representatives of subsection 5a & 5b. (A) *L. pumilum* (Jia 09001). (B) *L. davidii* (Du 11008). (C) *L. leichtlinii* var. *maximowiczii* (Du 11010). (D) *L. concolor* var. *pulchellum* (Du 11012). E, F, Representatives of subsection Leucolirion 6b. (E) *L. brownii* (Wang 11006). (F) *L. formosanum* (Du 12008). G, Representatives of subsection 5c (G) *L. taliense* (Jia 09002). H, I, Representatives of subsection Lophophorum III. (H) *L. sempervivoideum* (Liang and Lilium spp. Pollen in China (Liliaceae) PLOS ONE | www.plosone.org 17 January 2014 | Volume 9 | Issue 1 | e87841
addition, smaller pollen grains appear to survive better in habitats with extreme conditions.

Within the same species, pollen parameters from different provenances showed some variation (Table 1). Based on one-way ANOVA and LSD tests in 11 species (Table 2), results showed significant or extremely significant differences in pollen parameters in the same species from different habitats within the provenances, except for several single parameters in some species (Table 2). This may be explained as a consequence of interactions between phylogenetic and environmental constraints, as emphasized by Kawano and Kato [31].

Due to the wide variety of habitats in the distribution of *Lilium*, correlation analysis was performed to test which environmental factor is critical in influencing pollen parameters. The correlation analysis showed a significantly positive correlation between the polar axis and annual precipitation (P<0.05) and an extremely significantly positive correlation between the P/E ratio and annual precipitation (P<0.01) (Table 4). Hence, pollen size and shape showed a significantly positive correlation with annual precipitation. The absolute size of pollen grains influences harmomegathic mental conditions may be different types. For instance, *L. var. pulchellum* and *L. var. speciosum* show Martagon type pollen, whereas *L. dauricum* shows Martagon type pollen, and both occur in Northeast China under similar environmental conditions. Species native to the Himalayas and Hengdian Mountains show all of the pollen types within *Lilium*. The evolutionary trend of exine sculptures is likely not definitively correlated with pollen size.

**Conclusion**

In conclusion, we suggest recognising a new pollen type, *Formosanum*, to accommodate pollen from *L. formosanum*. Pollen sculpture patterns appear to reflect phylogenetic relationships and are useful for species or subsection delimitation within sections. The reduction and simplification evolutionary trend of pollen sculpture and size could be related to the selective pressure to better adapt to environment conditions, especially extreme environmental conditions. In addition, the evolutionary trends of exine sculpture and pollen size and shape are not definitively correlated. Pollen size and shape show a significantly positive correlation with annual precipitation. However, additional studies are required to confirm these evolution hypotheses in a broad sample of the genus using techniques such as transmission electron microscopy (TEM) to observe strata of the exine.

**Author Contributions**

Conceived and designed the experiments: GXJ YPD. Performed the experiments: YPD ZXW. Analyzed the data: YPD SL HBH. Contributed reagents/materials/analysis tools: YPD ZXW CW. Wrote the paper: YPD.

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