Abstract: Anthropogenic activities, such as the movement of plants through greening, can result in genetic disturbance that can interfere with local adaptation in wild populations. Although research is underway to prevent genetic disturbance associated with greening, genetic disturbance of intraspecific polyploidy, which is estimated to be present in 24% of vascular plants, has not been well studied. *Liriope muscari* is a polyploid complex with known diploid (2n = 36), tetraploid (2n = 72), and hexaploid (2n = 108) forms. The plants of this species tolerate dry and hot conditions and are therefore frequently used for greening and gardening. However, the distribution of this polyploid in Japan, its genetic structure, and genetic disturbance are not known. In this study, we investigated the polyploidy distribution and genetic structure in naturally distributed *L. muscari* in Japan using chloroplast DNA (cpDNA) haplotypes and nuclear DNA (nDNA). Commercially produced individuals were also studied and compared with natural populations to assess any genetic disturbance of the ploidy complex in this species. Chromosome counts, cpDNA, and nDNA results showed three genetically and cytologically distinct groups in Japan: first, a tetraploid group in mainland Japan; second, a hexaploid group in the Ryukyu Islands; and third, a diploid and tetraploid group in the Ryukyu Islands. Significant isolation by distance was also detected within the three groups (p = 0.001).

Genetic disturbance due to greening and gardening should be avoided among the three groups. Genetic disturbance can be reduced by using individuals derived from natural populations that are close to the sites used for greening and gardening. For commercially produced individuals, genetic disturbance is unlikely in the Kanto region, an area of high usage, while genetic disturbance is thought possible in the Ryukyu Islands.

Keywords: polyploidy; greening; human disturbance; MIG-seq; conservation
Polyploidy is common among plants; approximately 24% of vascular plants are polyploid [8]. Polyploidization may be harmful in the short term by causing karyotype instability and reducing opportunities for propagation, etc., but in the long term it is thought to play an important role in the creation of diversity and species differentiation. It can do this by reducing the manifestation of deleterious genes that can result from the redundancy of multiple genomes, enabling sexual differentiation and adaptation to different environments [9–11]. In plants with intraspecific polyploidy, each polyploid with its distinct characteristics is sometimes geographically limited to a specific habitat due to changes in ecological niches and reproductive isolation [12–14]. Despite the importance of polyploid plants in evolutionary processes, few studies on genetic disturbances in polyploid plants exist in the literature [15,16].

*Liriope muscari* (Decne.) Bailey is an evergreen perennial plant found in Mainland China, Taiwan, South Korea, and Japan. It is a polyploid species comprising three forms: diploid (2n = 36), tetraploid (2n = 72), and hexaploid (2n = 108) [17–21]. *L. muscari* is a useful plant with many applications and has been used as a herbal medicine since ancient times [22]. It is often used for greening because of its tolerance to drought and high temperature, and its high phytoremediation effect on Zn, Cu, Pb, Ni, and Cd in planted soil has been reported [23]. As of 2021, approximately 420,900 plants had been produced in Japan [24]. It is propagated by means of seeds from its hermaphrodite flowers. Its pollinators are not well documented, but visits by *Episyrphus* and *Parnara* are known (Watanabe pers. obs). Seeds are thought to be spread by birds [25], and the raccoon dog *Nctereutes procyonoides viverrinus* is known to feed on the seed [26]. As mentioned above, *L. muscari*, which is heavily used for greening and gardening, is likely to be subject to genetic disturbance. However, the distribution of the polyploids in Japan, its genetic structure, and genetic disturbance are not known.

Here, we report our investigations into the distribution of polyploidy and the genetic structure of naturally distributed *L. muscari* in Japan using chloroplast DNA (cpDNA) haplotypes and nuclear DNA (nDNA). We also investigate commercially produced individuals to compare with the natural populations and discuss the genetic disturbance of the polyploidy complex in this species.

2. Results

2.1. Polyploidy Level

The polyploidy level of 116 individuals at 86 sites were identified as follows (Table 1, Figure 1): 18 were diploid (8 sites), 61 were tetraploid (49 sites), and 37 were hexaploid (29 sites) (Table 1, Figure 1). We estimated the chromosome number using multiple individuals at 21 sites. The diploid individuals were found sporadically in the Ryukyu Islands and Taiwan, the tetraploids were found in mainland Japan and the southern Ryukyu Islands, and the hexaploids were found only in the central Ryukyu Islands. All 18 commercial individuals of *L. muscari* obtained from six nurseries in the Kanto region were tetraploid. Of the three individuals obtained from the nursery on the main island of Okinawa, two were tetraploid and one was hexaploid (Figure 2).

2.2. Chloroplast DNA Haplotypes

By combining the sequence data of four cpDNA regions that were analyzed, we identified nine haplotypes (m01, m02, m03, m04, m05, m06, m07, m08, and m09). The nucleotide substitutions and indels are shown in Table S1. The haplotype diversity (h) and nucleotide diversity (π) of *L. muscari* are shown in Table 2. TCS haplotype network based on a sequence with four regions combined showed that the central haplotype was m01. Haplotypes m02, m03, and m09 were confirmed as independent haplotypes, each with a single mutation step. Systematic connections were indicated between m04 and m05 and between m06, m07, and m08. In terms of the relationship between the haplotype network and the polyploids, of the nine haplotypes, a different polyploidy level was confirmed to be present within the same haplotype in m01, m03, and m07 (Figure 3b).
Table 1. Plant materials and localities of samples used in this study. Na: sample names, 2n: chromosome numbers, Hap: cpDNA haplotype names.

| Locality | Na       | 2n | Hap | Accession Numbers |
|----------|----------|----|-----|-------------------|
|          | cpDNA    | nDNA|
| Locality | Accession Numbers | Intron | trnT-trnL | trnL-trnF | atpB-rbcL |
| Natural distribution area | Mt. Mayumi, Hitachiota City, Ibaraki Pref. | wk270 | 72 | m09 | LC730913 | LC731014 | LC731095 | LC731176 | DRR412615 |
| Mainland Japan and Ryukyu Islands | Arakawa river, Nagatoro Town, Saitama Pref. | tm005 | 72 | m05 | LC730910 | LC731011 | LC731092 | LC731173 | DRR412612 |
| | Kamitanadare, Kisai Town, Saitama Pref. | wk018 | 72 |     | LC730911 | LC731012 | LC731093 | LC731174 | DRR412613 |
| | Sendabori, Matsudo City, Chiba Pref. | wk246 | 72 | m04 | LC730912 | LC731013 | LC731094 | LC731175 | DRR412614 |
| | Horiuchinai, Ichikawa City, Chiba Pref. | wk503 | 72 | m04 | LC730913 | LC731014 | LC731095 | LC731176 | DRR412615 |
| | Mogusa, Tama City, Tokyo Pref. | tm003 | 72 |     | LC730917 | LC731018 | LC731099 | LC731180 | DRR412619 |
| | Hané, Hamura City, Tokyo Pref. | wk546 | 72 | m09 | LC730913 | LC731014 | LC731095 | LC731176 | DRR412615 |
| | Motohachioji, Hachioji City, Tokyo Pref. | wk475 | 72 | m04 | LC730914 | LC731015 | LC731096 | LC731177 | DRR412616 |
| | Horiuchi, Hayama Town, Kanagawa Pref. | wk391 | 72 | m04 | LC730915 | LC731016 | LC731097 | LC731178 | DRR412617 |
| | Hakone, Hakone Town, Kanagawa Pref. | wk245 | 72 | m04 | LC730916 | LC731017 | LC731098 | LC731179 | DRR412618 |
| | Siokawa, Kani City, Gifu Pref. | tm198 | 72 | m09 | LC730917 | LC731018 | LC731099 | LC731180 | DRR412619 |
| | Imodani, Hashimoto City, Wakayama Pref. | tm006 | 72 | m09 | LC730918 | LC731019 | LC731100 | LC731181 | DRR412620 |
| | Hasemiya, Kimino Town, Wakayama Pref. | wk495 | 72 | m09 | LC730919 | LC731020 | LC731101 | LC731182 | DRR412621 |
| | Mt. Kurama, Sakyou Ward, Kyoto Pref. | wk278 | 72 |     | LC730920 | LC731021 | LC731102 | LC731183 | DRR412622 |
| | Mt. Takao, Kashiwara City, Osaka Pref. | tm008 | 72 |     | LC730921 | LC731022 | LC731103 | LC731184 | DRR412623 |
| | Higashiine, Akou City, Hyogo Pref. | wk062 | 72 | m09 | LC730922 | LC731023 | LC731104 | LC731185 | DRR412624 |
| | Asagoe, Okayama City, Okayama Pref. | wk067 | 72 |     | LC730923 | LC731024 | LC731105 | LC731186 | DRR412625 |
| | Mt. Ogonzan, Hiroshima City, Hiroshima Pref. | wk068 | 72 | m01 | LC730924 | LC731025 | LC731106 | LC731187 | DRR412626 |
| | Chuocho, Hikari City, Yamaguchi Pref. | wk073 | 72 | m01 | LC730922 | LC731023 | LC731104 | LC731185 | DRR412624 |
| | Chuocho, Hikari City, Yamaguchi Pref. | wk073 | 72 | m01 | LC730923 | LC731024 | LC731105 | LC731186 | DRR412625 |
| | Tyuzankei, Shimono-sede City, Yamaguchi Pref. | wk080 | 72 | m09 | LC730924 | LC731025 | LC731106 | LC731187 | DRR412626 |
| | Nagaonohana, Hagi City, Yamaguchi Pref. | tm012 | 72 |     | LC730925 | LC731026 | LC731107 | LC731188 | DRR412627 |
Table 1. Cont.

| Locality                        | Na    | 2n | Hap  | cpDNA                             | nDNA               |
|---------------------------------|-------|----|------|-----------------------------------|--------------------|
|                                 |       |    |      | *trnK'S* Intron  | *trnT-trnL* | *trnL-trnF* | *atpB-rbcL* |
| Onoyama, Sanyo Onoda City, Yamaguchi Pref. | wk024 | 72 | m01  | LC730926, LC731027, LC73109, LC731189 | DRR412628 |
| Kishinoue, Mannou Town, Kagawa Pref. | wk050 | 72 | m01  | LC730927, LC731028, LC731190 | DRR412629 |
| Sugeta, Ohzu City, Ehime Pref.  | wk165 | 72 | m04  | LC730927, LC731028, LC731190 | DRR412629 |
| Nagahama seashore, Hukuoka City, Fukuoka Pref. | wk009 | 72 | m09  | LC730928, LC731029, LC731110, LC731191 | DRR412630 |
| Onoyama, Sanyo Onoda City, Yamaguchi Pref. | wk010 | 72 |      |                  |          |
| Senbutudo, Kokura City, Fukuoka Pref. | tm014 | 72 |      |                  |          |
| Mt. Kagamiyama, Karatsu City, Saga Pref. | wk015 | 72 |      |                  |          |
| Hae, Tano Town, Miyazaki Pref.   | wk255 | 72 |      |                  |          |
| Okutsu, Kobayashi City, Miyazaki Pref. | wk256 | 72 |      |                  |          |
| Tatara, Ozu City, Kumamoto Pref. | wk134 | 72 |      |                  |          |
| Ino, Kikuchi City, Kumamoto Pref. | wk137 | 72 | m09  | LC730929, LC731030, LC731111, LC731192 | DRR412631 |
| Mt. Tokozan, Izumi City, Kagoshima Pref. | wk126 | 72 | m09  | LC730930, LC731031, LC731112, LC731193 | DRR412632 |
| Hatinarinjina, Kanoya City, Kagoshima Pref. | wk118 | 72 | m09  | LC730931, LC731032, LC731113, LC731194 | DRR412633 |
| Tomori, Amami City, Kagoshima Pref. | tm169 | 108|      |                  |          |
| Oazasetutsu, Amami City, Kagoshima Pref. | tm173 | 108| m08  | LC730932, LC731033, LC731114, LC731195 | DRR412634 |
| Mt. Amagi, Amagi Town, Kagoshima Pref. | wk182 | 108| m07  | LC730933, LC731034, LC731115, LC731196 | DRR412635 |
| Syoda, Tokunoshima Town, Kagoshima Pref. | tm022 | 108| m02  | LC730934, LC731035, LC731116, LC731197 | DRR412636 |
| San, Tokunoshima Town, Kagoshima Pref. | tm019 | 108| m02  | LC730935, LC731036, LC731117, LC731198 | DRR412637 |
| Kibirus, Wadomari Town, Kagoshima Pref. | wk548 | 108|      |                  |          |
| Tamirinazaki, China Town, Kagoshima Pref. | tm021 | 108|      |                  |          |
| Rikugidara, Izena Vil., Okinawa Pref. | tm020 | 108| m02  | LC730936, LC731037, LC731118, LC731199 | DRR412638 |
| Mt. Chizin, Izena Vil., Okinawa Pref. | tm168 | 108|      |                  |          |
| Mt. Gusuku, Ie Vil., Okinawa Pref. | tm086 | 108| m02  | LC730937, LC731038, LC731119, LC731200 | DRR412639 |
| Cape Hedo, Kunigami Vil., Okinawa Pref. | wk029 | 108|      |                  |          |
|                              | tm089 | 108|      |                  |          |
Table 1. Cont.

| Locality                                                                 | Na      | 2n  | Hap   | Accession Numbers |
|-------------------------------------------------------------------------|---------|-----|-------|-------------------|
|                                                                         |         |     |       | cpDNA             | nDNA              |
|                                                                         | tm037   | 36  | m06   | LC730939, LC731040, LC731121, LC731202 | DRR412641         |
|                                                                         | tm145   | 36  |       | LC730940, LC731041, LC731122, LC731203 | DRR412642         |
|                                                                         | tm146   | 36  |       | LC730941, LC731042, LC731123, LC731204 | DRR412643         |
|                                                                         | wk236   | 36  |       | LC730942, LC731043, LC731124, LC731205 | DRR412644         |
|                                                                         | wk237   | 36  | m06   | LC730943, LC731044, LC731125, LC731206 | DRR412645         |
|                                                                         | wk238   | 36  |       | LC730945, LC731046, LC731127, LC731208 | DRR412646         |
|                                                                         | tm149   | 108 |       | LC730946, LC731047, LC731128, LC731209 | DRR412647         |
|                                                                         | wk179   | 36  | m07   | LC730948, LC731049, LC731130, LC731211 | DRR412650         |
|                                                                         | tm031   | 108 | m02   | LC730949, LC731050, LC731131, LC731212 | DRR412651         |
|                                                                         | wk006   | 108 | m01   | LC730944, LC731045, LC731126, LC731207 | DRR412646         |
|                                                                         | wk007   | 108 | m01   | LC730945, LC731046, LC731127, LC731208 | DRR412647         |
|                                                                         | wk242   | 72  | m01   | LC730946, LC731047, LC731128, LC731209 | DRR412648         |
|                                                                         | wk020   | 108 | m02   | LC730947, LC731048, LC731129, LC731210 | DRR412649         |
|                                                                         | tm152   | 108 | m02   | LC730948, LC731049, LC731130, LC731211 | DRR412650         |
|                                                                         | tm153   | 108 | m02   | LC730949, LC731050, LC731131, LC731212 | DRR412651         |
|                                                                         | tm092   | 108 | m01   | LC730950, LC731051, LC731132, LC731213 | DRR412652         |
|                                                                         | tm095   | 108 | m01   | LC730950, LC731051, LC731132, LC731213 | DRR412652         |
|                                                                         | tm161   | 108 | m02   | LC730949, LC731050, LC731131, LC731212 | DRR412651         |
|                                                                         | tm150   | 36  | m01   | LC730950, LC731051, LC731132, LC731213 | DRR412652         |
|                                                                         | wk368   | 36  | m01   | LC730950, LC731051, LC731132, LC731213 | DRR412652         |
|                                                                         | tm151   | 108 | m01   | LC730950, LC731051, LC731132, LC731213 | DRR412652         |
|                                                                         | tm082   | 108 | m02   | LC730951, LC731052, LC731133, LC731214 | DRR412653         |
|                                                                         | wk295   | 108 | m02   | LC730952, LC731053, LC731134, LC731215 | DRR412654         |
|                                                                         | tm140   | 108 | m02   | LC730953, LC731054, LC731135, LC731216 | DRR412655         |
|                                                                         | tm142   | 108 |       | LC730954, LC731055, LC731136, LC731217 | DRR412656         |
|                                                                         | wk297   | 108 |       | LC730954, LC731055, LC731136, LC731217 | DRR412656         |
|                                                                         | wk292   | 108 | m02   | LC730954, LC731055, LC731136, LC731217 | DRR412656         |
Table 1. Cont.

| Locality | Na       | 2n  | Hap | Accession Numbers | Accession Numbers | Accession Numbers | Accession Numbers | Accession Numbers |
|----------|----------|-----|-----|-------------------|-------------------|-------------------|-------------------|-------------------|
|          |          |     |     | cpDNA             | nDNA              | trnK 5′ Intron   | trnT-trnL        | trnL-trnF         | atpB-rrnL         |
| Onosanrin, Miyakojima City, Okinawa Pref. | tm077    | 108 | m01 | LC730955          | LC731056          | LC731137          | LC731218          | DRR412657         |
|          | wk291    | 108 |     |                   |                   |                   |                   |                   |                   |
| Otakikoen, Miyakojima City, Okinawa Pref. | tm076    | 36  |     |                   |                   |                   |                   |                   |                   |
|          | tm078    | 36  | m07 | LC730956          | LC731057          | LC731138          | LC731219          | DRR412658         |
|          | tm079    | 36  |     |                   |                   |                   |                   |                   |                   |
|          | tm143    | 36  |     |                   |                   |                   |                   |                   |                   |
|          | wk298    | 36  |     |                   |                   |                   |                   |                   |                   |
| Nobarudake, Miyakojima City, Okinawa Pref. Umarezatonoutaki, Miyakojima City, Okinawa Pref. Mt. Makiyama, Miyakojima City, Okinawa Pref. | wk301    | 36  | m01 | LC730957          | LC731058          | LC731139          | LC731220          | DRR412659         |
| Kuninakautaki, Miyakojima City, Okinawa Pref. Toriike, Miyakojima City, Okinawa Pref. | wk334    | 108 |     |                   |                   |                   |                   |                   |                   |
| Misakiutaki, Ishigaki City, Okinawa Pref. Yutsun river, Taketomi Town, Okinawa Pref. | wk171    | 72  | m03 | LC730960          | LC731061          | LC731142          | LC731223          | DRR412662         |
|          | tm117    | 72  |     |                   |                   |                   |                   |                   |                   |
| Komi, Taketomi Town, Okinawa Pref. | wk309    | 72  | m01 | LC730961          | LC731062          | LC731143          | LC731224          | DRR412663         |
| Aira river, Taketomi Town, Okinawa Pref. Thindahanata, Yonaguni Town, Okinawa Pref. Agarizaki, Yonaguni Town, Okinawa Pref. Mt. Kubura, Yonaguni Town, Okinawa Pref. | wk311    | 72  | m03 | LC730962          | LC731063          | LC731144          | LC731225          | DRR412664         |
|          | wk312    | 72  |     |                   |                   |                   |                   |                   |                   |
| Nama seashore, Yonaguni Town, Okinawa Pref. Yonaguni, Yonaguni Town, Okinawa Pref. Taiwan Chingching-tsaoyan, Lanyu, Taitung Nurseries Nursery 01, Kawaguchi City, Saitama Pref. | wk211    | 72  | m03 | LC730965          | LC731066          | LC731147          | LC731228          | DRR412667         |
|          | wk212    | 72  | m03 | LC730966          | LC731067          | LC731148          | LC731229          | DRR412668         |
|          | tm098    | 36  | m03 | LC730967          | LC731068          | LC731149          | LC731230          | DRR412669         |
|          | wk460    | 72  | m05 | LC730968          | LC731069          | LC731150          | LC731231          | DRR412670         |
|          | wk461    | 72  | m04 | LC730969          | LC731070          | LC731151          | LC731232          | DRR412671         |
|          | wk462    | 72  | m04 | LC730970          | LC731071          | LC731152          | LC731233          | DRR412672         |
Table 1. Cont.

| Locality                                      | Na  | 2n  | Hap | Accession Numbers       |
|-----------------------------------------------|-----|-----|-----|-------------------------|
| Nursery 02, Kawaguchi City, Saitama Pref.     | wk63| 72  | m09 | LC730971, LC731072, LC731153, LC731234, DRR412673 |
|                                              | wk64| 72  | m04 | LC730972, LC731073, LC731154, LC731235, DRR412674 |
|                                              | wk65| 72  | m05 | LC730973, LC731074, LC731155, LC731236, DRR412675 |
| Nursery 03, Kawaguchi City, Saitama Pref.     | wk66| 72  | m05 | LC730974, LC731075, LC731156, LC731237, DRR412676 |
|                                              | wk67| 72  | m04 | LC730975, LC731076, LC731157, LC731238, DRR412677 |
|                                              | wk68| 72  | m04 | LC730976, LC731077, LC731158, LC731239, DRR412678 |
| Nursery 04, Yorii Town, Saitama Pref.         | wk66| 72  | m05 | LC730977, LC731078, LC731159, LC731240, DRR412679 |
|                                              | wk70| 72  | m04 | LC730978, LC731079, LC731160, LC731241, DRR412680 |
|                                              | wk71| 72  | m05 | LC730979, LC731080, LC731161, LC731242, DRR412681 |
| Nursery 05, Musashimurayama City, Tokyo Pref. | wk50| 72  | m09 | LC730980, LC731081, LC731162, LC731243, DRR412682 |
|                                              | wk51| 72  | m04 | LC730981, LC731082, LC731163, LC731244, DRR412683 |
|                                              | wk52| 72  | m09 | LC730982, LC731083, LC731164, LC731245, DRR412684 |
| Nursery 06, Chohu City, Tokyo Pref.           | wk72| 72  | m09 | LC730983, LC731084, LC731165, LC731246, DRR412685 |
|                                              | wk73| 72  | m09 | LC730984, LC731085, LC731166, LC731247, DRR412686 |
|                                              | wk74| 72  | m09 | LC730985, LC731086, LC731167, LC731248, DRR412687 |
| Nursery 07, Nishihara City, Okinawa Pref.     | wk97| 72  | m03 | LC730986, LC731087, LC731168, LC731249, DRR412688 |
|                                              | wk98| 72  | m03 | LC730987, LC731088, LC731169, LC731250, DRR412689 |
|                                              | wk99| 108 | m02 | LC730988, LC731089, LC731170, LC731251, DRR412690 |

Figure 1. Chromosomes at mitotic metaphase of three polyploidy levels of *L. muscarii*. (a): diploid (2n = 36, sample name: wk179), (b): tetraploid (2n = 72, sample name: tm179), (c): hexaploid (2n = 108, sample name: wk006). Scale bars are 5 µm.
Figure 2. Geographic distributions of polyploidy levels observed in *L. muscari*.

Table 2. cpDNA haplotype diversity and nucleotide diversity observed in *L. muscari*. N: number of samples, NH: number of haplotypes, h: haplotype diversity, π: nucleotide diversity.

| Groups             | Polyploidy | N  | NH | h     | π      |
|--------------------|------------|----|----|-------|--------|
| Natural distribution area |            |    |    |       |        |
| Group 1: Mainland Japan | 4x         | 24 | 3  | 0.583 | 0.00062 |
| Group 2: Ryukyu Islands | 6x         | 18 | 4  | 0.399 | 0.00030 |
| Group 3: Ryukyu Islands | 2x, 4x     | 18 | 4  | 0.739 | 0.00041 |
| Group 3–1          | 2x         | 8  | 4  | 0.821 | 0.00044 |
| Group 3–2          | 4x         | 10 | 2  | 0.533 | 0.00018 |
| Nurseries         |            |    |    |       |        |
| Nursery 01–06: Mainland Japan | 4x         | 18 | 2  | 0.471 | 0.00063 |
| Nursery 07: Ryukyu Islands | 4x         | 2  | 1  | 0.000 | 0.00000 |
| Nursery 07: Ryukyu Islands | 6x         | 1  | 1  | -     | -      |
\[ y = 8 \times 10^{-8} x + 0.032 \]
\[ y = 2 \times 10^{-9} x + 0.0338 \]

Figure 3. Geographical genetic structure of *L. muscari* using cpDNA and nDNA: (a) Neighbor-joining tree of *L. muscari*. Colored circles indicate differences in chloroplast DNA haplotypes. The numbers indicate the polyploidy level (2x: diploid, 4x: tetraploid, 6x: hexaploid). The color-coded areas indicate the three groups observed in the PCoA of the Mash distance. (b) Map of geographic distributions of haplotypes and polyploidy levels observed in *L. muscari*. Numbers indicate polyploidy level (2x: diploid, 4x: tetraploid, 6x: hexaploid). TCS network of 9 cpDNA haplotypes (m01, m02, m03, m04, m05, m06, m07, m08, m09) observed in cytotypes (2x, 4x, 6x). Each line connecting two haplotypes represents a single mutation step. Circles indicate sample size. The color-coded areas represent plots of individuals belonging to the three visually recognized groups. (c) PCoA analysis of Mash distance. The different colors of the plots indicate the chloroplast DNA haplotypes. The numbers on each axis indicate the percentage of variance. The color-coded areas represent plots of individuals belonging to the three visually recognized groups. (d) Correlation between Mash distance and geographic distance for the three groups observed in the PCoA of the Mash distance.

2.3. **Nuclear DNA**

The Mantel test for Mash distance to SNP-based genetic distance showed a significant correlation between data sets \((p = 0.001)\). SNP analysis finally called 93 SNPs with a genotyping rate of over 90%, and sequence coverage averages were above 30 for all individuals. Stacks parameters adjusted during SNP call settings were \(-m = 8, R0.85, -n = 1\). The statistics obtained from stacks-2.60 are shown in Table 3.

In the neighbor-joining tree output from Mashtree, three groups related to chloroplast DNA haplotypes and polyploidy were recognized: Group 1 includes mainland Japan and is tetraploid with major chloroplast DNA haplotypes m04 and m09; Group 2 is distributed in the Ryukyu Islands and is hexaploid with the major cpDNA haplotype m02; and Group 3, also distributed in the Ryukyu Islands, is diploid and tetraploid, with the major cpDNA haplotypes m01 and m03 (Figure 3a,b). Group 3 was identified as several closely related clusters composed of diploid and tetraploid (Figure 3a). PCoA plots also distinguished three groups (Figure 3c). Nuclear and chloroplast DNA results were generally consistent, but some discrepancies were observed. Haplotype m01 was found in all three groups, and
haplotype m07 was commonly found in groups 2 and 3 (Figure 3a). Mash distance and geographic distance in the three groups were significantly correlated ($p = 0.001$). In contrast to the trend of genetic diversity for cpDNA, a trend of increasing genetic diversity with increasing polyploidy level was observed for nDNA (Tables 2 and 3).

**Table 3.** nDNA Genetic diversity of *L. muscari*. N: number of samples, He: genetic diversity, Ho: observed heterozygosity, $F_{IS}$: inbreeding coefficient, $\pi$: nucleotide diversity.

| Groups                  | Polyplody | N   | He    | Ho    | $F_{IS}$ | $\pi$ |
|-------------------------|-----------|-----|-------|-------|----------|-------|
| Natural distribution area |           |     |       |       |          |       |
| Group 1: Mainland Japan  | 4x        | 24  | 0.00262 | 0.00283 | −0.00052 | 0.00268 |
| Group 2: Ryukyu Islands  | 6x        | 18  | 0.00377 | 0.00382 | 0.00061  | 0.00390 |
| Group 3: Ryukyu Islands  | 2x, 4x    | 18  | 0.00348 | 0.00256 | 0.00374  | 0.00360 |
| Group 3-1                | 2x        | 8   | 0.00178 | 0.00104 | 0.00294  | 0.00196 |
| Group 3-2                | 4x        | 10  | 0.00254 | 0.00299 | −0.00093 | 0.00262 |
| Nurseries               |           |     |       |       |          |       |
| Nursery 01-06: Mainland Japan | 4x    | 18  | 0.00254 | 0.00299 | −0.00093 | 0.00262 |
| Nursery 07: Ryukyu Islands | 4x    | 2   | 0.00234 | 0.00371 | 0.00018  | 0.00359 |
| Nursery 07: Ryukyu Islands | 6x    | 1   | 0.00301 | 0.00602 | 0        | 0.00602 |

**2.4. Genetic Characteristics of Commercially Produced *L. muscari***

The polyploidy, cpDNA haplotype, and nDNA characteristics of 18 *L. muscari* individuals obtained from nurseries 1–6 in the Kanto region were consistent with those naturally distributed around the nurseries. All were tetraploid, belonged to Group 1 identified by nDNA, and had cpDNA haplotypes m04, m05, and m09 (Figures 3b and 4). Of the three individuals obtained from the nursery on Okinawa Island, one was hexaploid and had haplotype m02 and was included in Group 2. The remaining two individuals were tetraploid, had cpDNA haplotype m03, and belonged to Group 3. One individual in Group 2 matched the major type obtained from the Okinawa mainland, while two individuals in Group 3 had cpDNA haplotype m03, a type with a more southerly distribution (Figures 3b and 4).

![Principal Coordinates (PCoA)](image)

Figure 4. PCoA of wild individuals and cultivars by Mash distance. The different shapes of the gray symbols represent the three groups identified in wild individuals. The colored symbols indicate differences in nurseries. Nurseries 01–06 are in two adjacent prefectures in the Kanto region of Japan (Saitama and Tokyo). Nursery 07 is in Okinawa Prefecture, Japan.
Genetic diversity of commercially produced *L. muscari* in nurseries in mainland Japan, with the main area of consumption near Tokyo, was comparable to the genetic diversity of Group 1 in mainland Japan in both cpDNA and nDNA (Tables 2 and 3).

3. Discussion

3.1. Distribution of Polyploidy Complex

All *L. muscari* individuals sampled from mainland Japan were tetraploid in our study; but the Ryukyu Islands samples comprised a mix of three polyploids. However, the hexaploid *L. muscari* has been reported in Hiroshima, mainland Japan [17]; the hexaploid form is therefore probably distributed throughout mainland Japan but only at a low frequency. Previous studies have reported the diploid form in Zhejiang Province, China; tetraploid in Korea; and hexaploid in mainland Japan [17,19,20,27]. Combined with the present results, there may be considerable overlap in the distribution of polyploidy, with the tetraploid distributed from the Ryukyu Islands to Taiwan and Zhejiang Province, China; the tetraploid from mainland Japan to the Ryukyu Islands and Korea; and the hexaploid from mainland Japan to the Ryukyu Islands. In our study we were able to clarify the polyploidy distribution pattern roughly in Japan. However, due to the limited number of survey sites and individuals, the distribution of *L. muscari* polyploidy throughout its distribution range remains unknown in term of the frequency of polyploidy in each region.

Two closely related diploid–tetraploid pairs were identified in the neighbor-joining tree by Mash distance, suggesting that the tetraploids have multiple origins. The multiple origins of polyploidy has also been reported elsewhere [28,29]. The multiple origins of polyploidy may be one of the factors contributing to the geographic obscurity of the distribution of the *L. muscari* polyploid complex in the Ryukyu Islands.

3.2. Genetic Structure of *L. muscari* in Japan

The three groups clearly differed in nDNA were also mostly consistent with the results for polyploidy and cpDNA, but partial discrepancies were observed in cpDNA. Incomplete lineage sorting and chloroplast capture are the main causes of mismatching between nuclear and chloroplast DNA [30–32]. In our present study, both processes are also possible; but given the wide distribution for haplotype m01, which was one of the two chloroplast DNA haplotypes that did not match the nuclear DNA results, and the fact that it was found in all three groups with different polyploidy, strongly suggests a high probability of incomplete lineage sorting.

The diverse results for polyploidy, chloroplast DNA, and nuclear DNA among the three groups suggest that a genetic barrier to gene flow exists between these groups. In general, geographic isolation and climatic conditions are known to be barriers to gene flow [33–35]. The adjacent Groups 1 and 2 are separated by the Strait of Tokara, which is known to have a different flora due to geographic isolation [35]. While Group 1 is tetraploid, Group 2 is hexaploid, and such differences in chromosome number are also a barrier to gene flow [36]. The boundary between Group 1 and Group 2 distributions is unclear, probably due to low sampling density, but multiple barriers may also prevent gene flow.

It is difficult to explain the geographic and climatic barriers to gene flow between Groups 2 and 3, whose distributions overlap in the Ryukyu Islands. Since Group 2 is hexaploid and Group 3 is diploid and tetraploid, a barrier to gene flow due to reproductive isolation at different polyploidy levels can be inferred. Group 3 includes diploid and tetraploid, but within the wild population the ploidy level is fixed to either diploid or tetraploid. Fixation of the polyploidy level in each local population suggests that diploid and tetraploid may be exclusive. It may be possible that gene flow is restricted between polyploids within Group 3.

The relationship between Mash distance and geographic distance showed a significant correlation for all groups. Restricted interbreeding between geographically distant individuals results in IBD [37]. *L. muscari* is a common species at low elevations within the range of our collection of samples. The possibility of restricted gene flow between diploids...
and tetraploids in Group 3 should be noted, but, in any case, it suggests that geographic proximity is important for interbreeding within groups.

We found lower cpDNA diversity of hexaploid individuals in the Ryukyu Islands compared to other polyploidy levels ($h = 0.399$, $\pi = 0.00030$). If both haplotype diversity and nucleotide diversity are low, the hexaploid may have experienced a more recent origin or a more restrictive bottleneck compared to other polyploidy levels. In either case, the hexaploid in the Ryukyu Islands is likely dependent on limited genetic sources. On the other hand, the higher genetic diversity of hexaploids compared to diploids and tetraploids in nDNA may reflect the increased diversity associated with genome duplication.

3.3. Taxonomic Confusion

The genus *Liriope* exhibits a certain amount of taxonomic confusion. *L. tawadae* is characterized by large plant size, broad and long leaf blades, and large flowers and long flower stalks, and has been reported in the Ryukyu Islands [38]. Due to the lack of morphological information, the relationship between *L. tawadae* and the result of this study is unclear. Some cpDNA haplotypes of *L. muscari* are shared with those of *L. spicata* (Watanabe, unpublished data), suggesting past hybridization. The situation is further complicated because *L. spicata* is known to be diploid, tetraploid, and hexaploid [19,21,39]. Further taxonomic reexamination, including related species, is therefore needed.

3.4. Potential of Anthropogenic Disturbance and Countermeasures

The commercially produced *L. muscari* was abundant near Tokyo; however, there was no obvious risk of genetic disturbance evident from our study. All *L. muscari* produced in nurseries near Tokyo were confirmed to be tetraploid and genetically close to naturally distributed individuals in the neighborhood. On the other hand, there is a possibility of genetic disturbance of *L. muscari* in the Ryukyu Islands due to greening. Despite our limited number of samples, we observed that genetically distinct *L. muscari* were being sold together. In addition, individuals from genetically distinct groups that were sold together also differed in their polyploidy levels, with one of the three individuals studied being Group 2 hexaploid and two being Group 3 tetraploids. The mixing of different polyploidy levels can cause additional problems. It has been noted that orthotopic growth of different polyploidy levels causes the eradication of minor polyploidy levels [40]. In fact, the polyploidy levels were fixed in populations where chromosome counts of multiple individuals were examined in this study. It should also be noted that pentaploids can easily be obtained by artificially crossing tetraploids and hexaploids from the Ryukyu Islands (Watanabe, unpublished data).

An effective means of preventing anthropogenic genetic disturbances of *L. muscari* is to avoid contact between genetically distinct groups. By selecting *L. muscari* that belong to the same genetic group as the natural population surrounding the proposed greening site, and by using seed collected from a single group during seedling production, contact between genetically distinct groups can be reduced. Considering within groups, genetic distance between geographically close individuals is close, so using individuals derived from natural populations that are geographically close to the proposed greening site is expected to further reduce genetic disturbance. Group 3 suggests multiple origins of the tetraploid, and although the situation may be complex, it is expected that the supply of individuals with the same polyploidy level from a natural population near the proposed site at the time of greening will reduce genetic disturbance.

Determining a level of genetic disturbance based on genetic information remains difficult for greening officials. With *L. muscari* in Japan, it is possible to recognize groups that should avoid contact with each other based on their geographic distribution and polyploidy levels. Three groups in Japan should avoid being genetically disturbed. The first group is the tetraploid distributed in mainland Japan. There are also two groups in the Ryukyu Islands that have overlapping geographic boundaries but can be distinguished by polyploidy level. The second group is the hexaploid of the Ryukyu Islands. The third
group is the diploid and tetraploid of the Ryukyu Islands. At present it is difficult to estimate the polyploidy level of *L. muscari* by morphological features, but accumulating such morphological information will be an effective way to test for correspondence with the polyploidy level in order to aid their recognition in the field.

4. Materials and Methods

4.1. Collection of Materials

We collected between one and five individuals from 86 sites (totaling 116 individuals) in the natural distribution area, ranging from Niigata Prefecture in Japan to Taiwan. A further 21 commercially produced individuals were also collected: three each from two nurseries in Tokyo, four nurseries in Saitama Prefecture, and one nursery in Okinawa Prefecture (Table 1).

4.2. Determination of Polyploid Level

For each of the 137 *L. muscari* individuals collected, we counted their chromosome number using the aceto-orcein squash method. The root tip meristems were placed in a 0.002 M 8-hydroxyquinoline solution and pretreated at room temperature for 4–5 h. Subsequently, they were left in acetic acid alcohol (3:1, anhydrous ethanol–glacial acetic acid) to harden for at least five hours at 4 °C. After hardening, they were disaggregated for approximately 40 s in a 60 °C disaggregation solution (2:1, 1N hydrochloric acid–45% acetic acid) and stained for 1 to 15 min in a 2% aceto-orcein solution, and then squashed on a glass slide. The number of chromosomes was counted in somatic cells at metaphase.

4.3. Chloroplast DNA Analysis

The total DNA was extracted following the CTAB method of Doyle and Doyle (1987) after removing polysaccharides using the method of Setoguchi and Ohba (1995) [41,42].

Using four pairs of primers developed by Taberlet, (1991), Denda and Yokota (2003), Nakamura et al. (2006), Liston and Kadereit (1995) [43–46], polymerase chain reaction (PCR) amplification was conducted for the following intron and intergenic spacers and regions: *trnK* 5′ intron: (5′-CTCAACGTTAGTACTCG-3′, 5′-CCAAAAACTCCACAGGTTCG-3′), *trnT-trnL*: (5′-GCGATGCTCTAACCTCTGAG-3′, 5′-TAGCGTCTACCGATTTCCG-3′), *trnL-trnF*: (5′-ATTTGAACTGGTGACACGAG-3′, 5′-ATTTGAACTGGTGACACGAG-3′), and *atpB-rbcL*: (5′-ACTTAGAGGAGCTCCCGTGTACATCTCC-3′, 5′-GAGTTACTCCGAGATGTGCG-3′) intergenic regions. PCR amplification was performed using a PCR Thermal cycler SP (Takara), and base sequence determination was performed using a CEQ 8800 capillary DNA sequencer (Beckman Coulter). The base sequence obtained in this manner was aligned using the default parameters of the ClustalW program implemented in the MEGA X software [47]. The chloroplast DNA haplotypes were detected based on the arrangement of 3021 bases for the four combined domains. In addition, using DnaSP version 6.12.03 [48], the haplotypic diversity (h) and nucleotide diversity (π) were calculated for each polyploid and each region in which differences in polyploid distribution had been determined [49]. A parsimony haplotype network diagram of chloroplast DNA was created using the PopART 1.7 TCS network based on the data set in which the base sequences for the *trnK* intron, the *trnT-L, trnL-F* and *atpB-rbcL* intergenic regions [50], and the *matK* gene region were combined. Insertion–deletion (INDEL) mutations were excluded from analysis on the TCS network, so a dataset was created with INDELS replaced with base substitutions. We excluded from the analysis all repeated insertion–deletion of sequences for which the homology of the mutations was unclear.

4.4. Nuclear DNA Analysis

Nuclear DNA was investigated by sequencing with MIG-seq analysis [51,52], which creates a reduced library of genomes for samples with known chromosome numbers and chloroplast DNA haplotypes. The region flanked by SSRs was PCR amplified using 8 primers in the first PCR, and the resulting amplicons were indexed in a second PCR.
The indexed DNA library was sequenced using the MiSeq Reagent Kit v3 (150-cycle) (Illumina, San Diego, CA, USA). The obtained reads were filtered using Trimmomatic 0.39 [53]. Adapter sequences were removed using default settings and short reads were removed (MINLEN:79). Low quality reads were then removed and trimmed (SLIDING-WINDOW:4:15, CROP:79). To investigate genetic distances between individuals, we used Mashtree [54] to create a neighbor-joining tree based on Mash distance between individuals. Mashtree parameters were k-mer length 21 (--kmerlength 21), sketch k-mer count was set to 30,000 (--sketchsize 30,000), and k-mers with a count of less than 2 were excluded from the analysis (--mindepth 2).

The genetic analysis of polyploid data involves certain challenges. The main one is the difficulty in estimating allele frequencies of the polyploid. Many existing analysis methods require allele doses, for example, hundreds of coverages to recover tetraploid alleles with 90% confidence [55]. It is difficult to obtain this amount of information by normal greening. k-mer analysis using the MinHash method does not require allele dosage, thus alleviating the difficulties of polyploid analysis [56]. However, Mashtree-derived Mash distances provide information equivalent to genetic distances. They are obtained by evaluating the similarity of reads resolved into k-mers. To assess the similarity between individuals, Principal Coordinate Analysis (PCoA) using Mash distance was performed in GenAlex 6.502. Mantel tests were performed on GenAlex 6.502 for Mash distance and geographic distance to estimate isolation by distance (IBD) between the groups recognized by PCoA (Tables S2–S4). However, such data should be treated with caution when evaluating between different polyploidy levels using Mash distances, as they can be subject to bias [56]. To assess the plausibility of comparisons between different polyploidy levels using the Mash distance, a Mantel test was performed on the genetic distance obtained from single nucleotide polymorphisms (SNP) and the Mash distance (Table S5). The Mantel test was performed using GenAlex 6.502, and the SNP call was made using the denovo_map.pl pipeline from stacks-2.60 [57]. The parameters for estimating the genetic diversity of _L. muscari_ were also obtained with stacks-2.60. Stacks is an analytical protocol for a diploid model, which is usually difficult to apply to a polyploid. Although several informative alleles are lost, stacks can be used to analyze polyploidy by treating them as diploid by linking copies derived from polyploidy [58].

5. Conclusions

In Japan, there are three groups of natural populations of _L. muscari_ recognized by polyploidy, cpDNA, and nDNA: the first is the mainland tetraploid group; the second is the Ryukyu Islands hexaploid group; and the third is the Ryukyu Islands diploid and tetraploid group. For the reduction of potential risks regarding the destruction of the local adaptation of natural individuals around the greening area and for the establishment of the planted individuals, genetic disturbance associated with greening between these three groups must be avoided. In the Kanto region near large cities, the possibility of genetic disturbance due to greening is low because the cultivated products and the surrounding natural populations belong to the same group. On the other hand, in the Ryukyu Islands, individuals belonging to different groups were being sold in the same nursery, suggesting the possibility of genetic disturbance between groups due to greening. Within the three groups, distinct IBD could be identified in nDNA. Using individuals derived from natural populations that are geographically close to the proposed greening site is expected to further reduce genetic disturbance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11223015/s1, Table S1: Nucleotide substitutions and indels observed in the _Liriope muscari_; Table S2: Geographic distance (below) and Mash distance (above) for Group 1; Table S3: Geographic distance (below) and Mash distance (above) for Group 2; Table S4: Geographic distance (below) and Mash distance (above) for Group 3; Table S5: Mash distance (below) and SNP-based genetic distance (above) for all individuals.
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