An evolutionary biological study of the subfamily Lemnoideae (Araceae) with special reference to the genus *Lemna* in East Asia

ウキクサ亜科（サトイモ科）の進化生物学的研究－特に東アジア産アオウキクサ属 *Lemna*を中心として

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TABLE OF CONTENTS

SUMMARY

Aquatic plants are a polyphyletic plant group that depend on the aquatic environment for part of their life history. Each of these plants is considered to have adapted and speciated into a waterside environment; however, evolutionary and taxonomic research has been stagnant because of the difficulty in investigation and observation compared with that in general terrestrial plants. *Lemna* L. (Araceae, Lemnoideae) is a good model plant for aquatic botanical research. It is the largest genus in the subfamily Lemnoideae and is a tiny floating aquatic plant that is widely distributed throughout the world, except in the polar areas. Because *Lemna* species have simple organs and their flowers are rare, identification is difficult, and taxonomic challenges remain. Therefore, in the present study, the genetic diversity, morphology, floral morphology, interspecific hybridization, and distribution of *Lemna* and their relationship with evolution and speciation are discussed in five chapters.

In Chapter 1, I summarize the taxonomic challenges, relationships between evolution and species differentiation, genetic diversity, morphological characteristics, flower morphology, and interspecies hybridization of *Lemna*.

In Chapter 2, I examined whether the physiological and morphological characteristics were phenotypic plasticity or a reflection of species differences in *Le. aequinoctialis* Welw. and *Le. aoukikus*a Beppu et Murata. Physiological analysis of 11 strains revealed two types of flowers: protogynous and homogamous. Physiological group A, which was protogynous, was self-incompatible, and mature seeds could not be obtained. In contrast, mature seeds were obtained from group B, which was homogamous. These two groups were morphologically different, consistent with the characteristics of *Le. aequinoctialis* and *Le. aoukikus*a, respectively. Chloroplast DNA-based phylogenetic analyses also supported the division of these two groups, and it was concluded that *Le. aequinoctialis* and *Le. aoukikus*a are independent taxa.

In Chapter 3, I estimated the clonality of the Japanese *Le. trisulca* L. based on genome-wide Single-nucleotide polymorphisms (SNPs) data, clarified the population structure, and evaluated the relationship between morphology (root presence and number of frond veins) and genetic features. Based on the genetic distance calculated for three

relationships, which are “within individual”, “among individuals in the same population”, and “between population”, there was no significant difference in genetic distance between “within individual” and “among individuals in the same population” relationships. This suggests that each population is usually maintained by the clonal reproduction of a single plant, that each population does not share the same individual, and that migration among populations is likely caused by seeds. This seems to be because submerged plants have a low tolerance to drying conditions, making it difficult for leaflets to disperse long distances. No relationship was detected between the morphological diversity and genetic characteristics in the presence frond veins or their number.

In Chapter 4, to clarify the relationship between interspecific hybridization, differentiation of morphological traits, and speciation in *Lemna*, I investigated physiological and morphological variation, pollen germination rate, and phylogenetic relationships for Japanese *Le. minor* L., *Le. japonica* Landolt, and *Le. turionifera* Landolt in section *Lemna*. Chloroplast DNA analysis yielded three haplotypes and clustering analysis based on genome-wide SNP data revealed three genetic clusters in the three Japanese species.

As flower characteristics of *Le. japonica* have not been sufficiently reported, I could not identify the actual *Le. japonica*. Based on *Le. japonica* type material (7182) observation and comparison, cluster B (haplotype J), which has a light reddish spathe and never an open anther, was classified as *Le. japonica*. Subcluster a (haplotype M), with a transparent spathe, was identified as *Le. minor*. Cluster C (haplotype T), with strong reddish spathe and dehiscent anthers, was identified as *Le. turionifera*. There was only one substitution between haplotypes J and M; however, there were 16 and 15 substitutions within haplotypes J and T, M and T, respectively. The three species were also morphologically distinguished in Principal Component Analysis (PCA), and the variances in frond number, frond thickness, ovary diameter, and stigma width were high. In the estimation of population demographic history, the scenario in which *Le. minor* and *Le. turionifera* hybridized approximately 6,850 (2,100–13,100) generations ago was most highly supported, and the possibility that *Le. japonica* originating from hybridization, was supported, as previously reported.

In the pollen germination rate test, putative parental species *Le. turionifera* had a significantly higher germination rate than *Le. minor*, whereas pollen from *Le. japonica*

seeds did not germinate. These results suggested that the combination of the putative parental species *Le. minor* as the female seed parent and *Le. turionifera* as a male pollen donor, as deduced from chloroplast DNA analysis. Additionally, the distribution of *Le. minor* and *Le. japonica* was divided into Northern and Southern Japan, which may reflect the seed-formation ability of the two species.

In Chapter 5, I report two new distributions of the Lemnoideae plants (*Landoltia punctata* and *Le. turionifera*) and their habitat information to date based on field observations.

CHAPTER 1

General Introduction

Aquatic plants are defined as plants that growing in water, saturated or waterlogged soils (Cowardin et al. 1979; Tiner 2012). They play an important role in aquatic ecosystems. The functions of aquatic food chains, nutrient regeneration, and air saturation have been widely studied in aquatic ecosystems (Gettys et al. 2014; Harrison and Mann 1975; Pokorný and Květ 2003; Raven 1995; Teal 1962).

Hydrophytes, which occupy 2 % in angiosperm, is known as the ancestor of angiosperm families (Arber and Arber 1920; Coiffare et al. 2007; Cook 1999; Soltis et al. 2008). Their main habitat, aquatic environment is not stable, the plants have faced difficulty to survive in the extreme conditions since they have been established around 115–125 million years ago (Soltis et al. 2008). In the water circumstance, insufficient light and carbon dioxide restrict photosynthesis, diverse water types (clarity, salinity) influence aquatic plants (Lacoul and Freedman 2006; Pokorny et al. 1984). These various factors allowed the hydrophytes adopt and develop themselves to survive in the water environment. As the representative characteristics of aquatic plants, their phenotypic plasticity and reproductive system were well-reported (Dorken and Barrett 2004; Kaplan 2002; Riis et al. 2010; Well and Pigliucci 2000). Due to their ecological importance, it is very important to have a deep understanding of them, however, aquatic plants were historically less studied than terrestrial plants (Croft and Preston 1996, Marechal 2019).

Most typical aquatic plants (floating, submerging plants) can propagate asexually, even the asexual propagation capacity is more prevalent than sexual propagation (Philbrick and Les 1996). Their variable morphological and physiological characteristics make taxonomy and understanding them not easy. Considering these characteristics of aquatic plants, it would be appropriate to observe them in the same environment to deeply understand them. But considering the size and growth speed of common aquatic plants, such as water lily (*Nymphaea* L.) and yellow water lily (*Nuphar* Sm.), it is not a simple

issue.

Subfamily Lemnoideae (Araceae), the smallest angiosperm, is one of the most representative aquatic plants. Five genera (*Landoltia* Les & Crawford, *Lemna* L., *Spirodela* Schleid., *Wolffia* Horkel ex Schleid., and *Wolffiella* Hegelm.) belong to Lemnoideae (Bog et al. 2020b: as Lemnaceae). It freely floats on (or under) water surface, and widely distributes throughout the world except for the polar and desert regions. It frequently lives in rice field, pond, lake, wetland, or stream.

The largest genus in Lemnoideae, *Lemna* includes 12 to 14 taxa in the world (Beppu et al. 1985; Bog et al. 2020b), two species, and eight species with two subspecies have been recorded to South Korea and Japan, respectively (Kadono 2014; Kim et al. 2017). Identification of the *Lemna* species has been treated a challenge for over a century because they mainly propagate asexually and have phenotypic plasticity. Their rarity of natural flower, extremely reduced organs, variable morphology, natural hybridization made difficult to understand each species in morphology, physiology, ecology, or detect relationships between some close species (Landolt 1986). Furthermore, whether their various characteristics reflect genetic characteristics and whether they represent distinct species has been difficult to examine in depth.

Lemna species, their tiny body size let them be cultivated in laboratory, rapid growth rate under the right conditions had made them used as model plants in various fields (Acosta 2021). Thus, I chose genus *Lemna* as a suitable material for understanding aquatic plants.

Genus *Lemna* in Japan, the endemic two taxa, *L. aoukikusa* Beppu et Murata and *L. aoukikusa* subsp. *hokurikuensis* Beppu et Murata are treated as the synonym of cosmopolitan species, *L. aequinoctialis* Welw. The physiological and morphological key characters were not carefully observed except for the nomenclator Beppu et al. (1985), they needed to be observed carefully by cultivating under the same conditions. In addition, it was also necessary to check whether different characteristics reflected genetic information. In Chapter 2, I aimed to prove whether *L. aoukikusa* is same or different with *L. aequinoctialis*.

Lemna species, like any aquatic plant, usually keep their populations as clone (Chmilar and Laird 2019; Landolt 1986). In particular, the flower and seed of *L. trisulca* have never been reported in Japan, and how *Lemna* species disperse to hydrologically not

connected area was only confirmed by checking simulation of frond hydration (Ridley 1930). In Chapter 3, I aimed to confirm whether all *L. trisulca* in Japan were originated from clones based on the molecular data, and whether their morphological variation was due to plasticity or genetic variation.

Meanwhile, natural hybridization is considered a good way to evolve plants which usually reproduce through mitosis rather than meiosis, such as aquatic plants (Arnold 1997; Les and Philbrick 1993). It is reported that frequent hybrid formation occurs in section *Lemna* (genus *Lemna*). Three native species, *L. minor* L., *L. japonica* Landolt, *L. turionifera* Landolt distribute to Japan. Their identification usually relies on vegetative characteristics, their floral characters were still not studied sufficiently. The vegetative characters are variable, and their taxonomy is also problematic. The claim that *L. japonica* is a hybrid of *L. minor* and *L. turionifera* has been known by several researchers, but no study has been conducted yet on when, how, or where they hybridized. In Chapter 4, I aimed to conduct their taxonomic identification accurately based on molecular studies and consider the relationship between the species through morphological and physiological comparison based on *L. japonica*'s type strain checking. In addition, because subfamily Lemnoideae was not sufficiently studied in Korea, I also tried to explore unknown Korean Lemnoideae species during conducting this study. I hope this study, conducted on duckweed (Lemnoideae), the world's smallest angiosperm plant, will can help to understand aquatic plants more deeply.

CHAPTER 2

Two lineages in *L. aequinoctialis sensu lato* based on physiology, morphology, and phylogeny

2.1 INTRODUCTION

The subfamily Lemnoideae (Araceae), floating or submerging monocotyledonous aquatic plant is known as the smallest angiosperm in the world (Acosta et al., 2021; Landolt, 1986; Wang et al., 2010; Xu et al., 2015). Having five genera and 36 species, Lemnoideae is distributing worldwide, especially in tropical and subtropical regions (Bog et al., 2020a; Landolt, 1986; Les 2020). They flower rarely, mainly expand their population size lean on fast clonal propagation.

The largest genus in Lemnoideae, *Lemna* L. is characterized by having solitary root per one frond, and one pistil with two stamens which are surrounded by a spathe. Owing to their 0.6−9 mm of small size (except for *Lemna trisulca* L.), simple, and variable organs, it is hard to identify them, and it caused a problem limiting their taxonomical boundary and status.

Lemna aequinoctialis Welw. is one of the most widely spreading species in the genus *Lemna* (Landolt, 1986). The species has been widely studied by many scientists in diverse fields. Taxonomy, genetic diversity, and various responses to environmental control have been reported for over half a century (Kaihara et al., 1981; Khurana and Maheshwari 1983; Khurana et al., 2011; Kondo et al., 2007; Landolt 1957; Tang et al., 2015; Xu et al., 2015). Most of recent molecular studies imply that *L. aequinoctialis* is monophyletic species (Bog et al. 2010; Braglia et al. 2021a; Kim et al. 2017; Neto et al. 2019), besides, the two types of flowers, 1) homogamous and self-compatible, 2) protogynous and self-incompatible, have been reported in *L. aequinoctialis* group in Japan (Beppu 1981).

Beppu et al. (1985) established two new Japanese endemic taxa, *Lemna aoukikusa* Beppu et Murata and *L. aoukikusa* subsp. *hokurikuensis* Beppu et Murata differed from *L. aequinoctialis* based on their physiological, morphological, cytological, ecological,

and molecular (allozyme) studies (Beppu 1981; Beppu and Takimoto 1981a, b, c; Yukawa and Takimoto 1976). Nevertheless, the physiological features they claimed to be the greatest features of the new taxa have been sometimes dismissed as variations limited only within Japan due to the thousand years of rice cultivation (Landolt 1986).

Floral mechanisms are well known as a crucial factor in speciation (Coyne and Orr 2004; Mayr 1947). In the genus *Lemna*, floral mechanisms and morphology may increase their genetic diversity and stimulate evolution and speciation. However, flowers of *L. aequionctialis* are small, rarely, and has a short flowering period, and it make difficult to observe its floral mechanisms. In situations where most of the *Lemna* species except *L. aequinoctialis* are reported to be protogynous and self-incompatible (Les 2020), the discovery of two flowering characteristics in one species (*L. aequinoctialis* group in Japan; especially within *L. aequinoctialis s.l.* we have treated the two taxa, *L. aequinoctialis sensu* Beppu et al. (1986) and *L. aoukikusa* as *L. aequinoctialis* complex, here) needs to be confirmed based on detailed observation, and the possibility of speciation can be considered with various insight.

Beppu et al. (1985) reported morphological difference between *L. aequinoctialis* group in Japan, but their extreme similarities did not appeal agreement of their taxonomy from other scientists (Halder and Venu 2012; Landolt 1986; Sree et al. 2016). In particular, Landolt (1986) pointed out a limitation of the study of Beppu et al. (1985) that only Japanese *Lemna* plants were compared. Therefore, it is recognized the necessity of abroad plants observation and comparison.

Since Wang et al. (2010) suggested the DNA barcoding for Lemnoideae identification, the recent *Lemna* researchers commonly reflect the DNA barcoding region(s) in their research results (Braglia et al. 2021a,b; Chen et al. 2022; Marconi et al. 2019; Suzuki et al. 2014), but it is difficult to get the report of Japanese *L. aequinoctialis* group (Borisjuk et al. 2015). Considering that genes reflect various biological characteristics (Hamrick et al. 1979), the molecular-based biological characteristics can be a good method to weigh their taxonomy.

Since Lemnoideae species are considered economically valuable plants due to their variety of utilities (Appenroth et al. 2017; Chakrabarti et al. 2018; Devlamynck 2021; Fourounjian et al. 2020; Iqbal et al. 2019; Rahmani et al. 1999), their taxonomy and identification is very important. Owing to the high diversity in *L. aequinoctialis*, the first

objective of this study was to check what various properties are observed within the *L. aequinoctialis sensu lato* and grouping the observed various properties and measure the difference between groups. The second objective was detecting whether the gene reflects the difference of groups. The third objective was considering whether the groups in *L. aequinoctialis* complex have difference in geographical distribution, and whether their distribution was influenced by some physiological properties. In addition, I aimed to consider that whether these situations also occur out of Japan. Finally, I aimed to determine whether the representative traits of groups in *L. aequinoctialis* complex contribute to treating the groups at the species level. Hence, the results of physiological, morphological, and molecular analyses were fused to investigate the relationship between *L. aequinoctialis* complex.

2.2 MATERIALS AND METHODS

2.2.1 Sample colletion

From April 3rd in 2019 to October 4th in 2020, samples of *L. aequinoctialis* complex were collected from 70 localities of rice field, lake, and pond in Japan, Korea, and Thailand (Table 2.1). In addition, I used Landolt's strain of *L. aequinoctialis*, strain number (no.) 6746, which has been used previously for several studies, such as cytology, molecule, morphology, physiology, and taxonomy of *Lemna* (Beppu et al. 1985; Hillman 1959; Hoang et al. 2019; Kandeler and Hügel 1974; Khurana et al. 2011; Landolt 1957, 1986; Tanaka et al. 1997; Wang et al. 2010). *L. valdiviana* Phil. was also collected in Japan for the phylogenetic analysis. The voucher specimens (Table 2.1) were stored in the herbarium of Niigata University Herbarium (NGU) or Andong National University Herbarium (ANH).

2.2.2 Establishment of axenic strain

More than 12 fronds from each locality were sterilized with 0.5 % of NaClO for 10 minutes, and washed by autoclaved distilled water for 10 minutes twice in a clean bench. The successfully sterilized single frond was cultured on semisolid (0.45–0.8 % agar) Hutner's medium (Hutner 1953) with added 1 % sucrose at pH 6.5 in disposable petri dish (90 mmφ × 20 mm). The cultures were conduted at 25 ℃ under 12L:12D photoperiodic light from LED lamps (PF20-S9WT8-D, Nippon medical & Chemical

instruments, Osaka, Japan) at 40 ± 10 μmol m^{-2} s^{-1} as the surface light intensity of the petri dish in the incubator (MIR-253, SANYO, Gunma, Japan). In this culture condition, the strains mainly propagate clonally by means of frond division. One representative strain of each locality was established and named with NGY (Niigata University Yuri) in this study (Table 2.1).

2.2.3 Flower inducing

In order to observe the flower development and self-fertilization ability of *L. aequinoctialis* complex, I attempted flower inducing for the 13 strains (NGY14, 33, 59, 122, 123, 128, 140, 142, 178, 180, 196, 302, and 6746) based on the modified method of Kaihara et al. (1981) as described below. Fronds from a clonal strain were pre-cultured in the 20^{-1} strength nitrate-free Hutner's medium (with 1 % sucrose) with added 10 μM Benzoic acid in 100 ml flask for 10 days. To make nitrate-free condition, NH_4NO_3 was replaced with an equimolecular amount of KNO_3. The 15–22 fronds bearing flower primordia per strain were numbered and transplanted to the same of agar-semisolid (0.3 %) medium in 90 × 20 mm sized petri dish to monitor the flower development (Fig. S2.1). These cultures were conducted at 25 ℃, under 40 ± 3 μmol m^{-2} s^{-1} of continuous light from both LED and fluorescent light (Terukuni Denki, Tokyo, Japan) in incubator (LH-240N, Nippon medical & Chemical instruments, Osaka, Japan).

In order to perform the morphometric analysis including flower morphology, I induced flowers on the same condition as mentioned above, but in liquid medium. I also attempted the flower inducing of 10 strains (NGY14, 33, 53, 59, 66, 122, 123, 128, 140, 302) in the other way which is based on the photoperiodic light treatment (Landolt 1986; Beppu et al. 1985; Hillman 1975). I offered eight or 12 hours of light. In this treatment the plants were cultured in liquid Hutner's medium (including 1 % sucrose) in 100 ml flask for over 14 days.

2.2.4 Observation of flower development and self-fertilization ability

Totally 495 flowering fronds (Table 2.2) from 11 strains were monitored every 12 hours for seven days under dissecting microscope (SZ2-ILST, Olympus, Tokyo, Japan). I recorded each frond's flowering moment, and at the final day of observation, I finally judged which flower organ matured earlier. New flowers which developed on new fronds

during monitoring were also observed. While the observation, the frond number which bears fruit was counted. In addition, dropped seed number was recorded to count the frond number which successfully matured at the last day.

2.2.5 Morphometric analysis

The flowers that were induced in two ways did not show significant morphological differences based on the method, so all of them were used for morpholometric analysis. For morphometric measurement, totally 310 flowering plants from the 13 strains were measured in fresh or immersed (70 % of ethanol) condition (Table S2.2). To observe them in similar environment to natural condition, all investigated plants were cultured on the liquid medium. I measured previously quoted 15 quantitative characters by Beppu et al. (1985) and Landolt (1986), as well as eight characters which could potentially distinguish for *L. aoukikusa* from *L. aequinoctialis* complex. In addition, I focused two qualitative characters such as the shapes of root and root tip, because Beppu et al. (1985) has suggested that *L. aequinoctialis* complex can be divided by three types based on these characters. The root characters were observed from the longest one. For the sizes of the anther and its filament, the longer one of two stamens was measured. The measured characters were statistically tested by Chi-Square test, Mann-Whitney Wilcoxon test, or T-test (Student's test, Welch's test) to reveal the significance between physiologically divided two groups.

To analyze morphological relationship among physiological groups, a principal component analysis (PCA) was performed. I excluded incompletely measured plants due to early-wilted some organs. Among the 23 characters, I selected the characters having high correlation for each of the principal factors, and then, removed characters which were highly correlated (which had the value over 0.7) to each other in covariability. Finally, the six quantitative characters (spathe length, spathe diameter, stigma width, ovary length, filament diameter, and anther length) were selected and 191 fronds were used for PCA. All statistical tests and PCA were performed using JMP version 11.2.0 (SAS Institute, Inc., Cary, NC, USA).

2.2.6 Phylogenetic analysis

Total DNA was extracted from a living frond via the cetyltrimethylammonium

bromide (CTAB) method (Milligan 1992). The all-partial DNA sequences was amplified through polymerase chain reaction (PCR) using a TaKaRa PCR Thermal Cycler (TP650 Standard, TaKaRa Bio Inc., Kusatsu, Japan). The reactions were conducted with 0.8 µl of extracted DNA and 0.06 µl of each of the primers. 15 µl of EmeraldAmp™ PCR Master Mix (Takara Bio Inc.) was added and the final volume of mixture was 30 µl. The primer information and PCR conditions are presented as follows. The noncoding spacer region, *atpF−atpH* was amplified with the primers, *atpF* (5′-ACTCGCACACACTCCCTTTCC-3′) and *atpH* (5′-GCTTTTATGGAAGCTTTAACAAT-3′), which were described by Fazekas et al. (2008). The amplification was conducted as initial denaturation for 5 min at 95 °C, 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 51 °C, extension for 1 min at 71 °C, and final extension for 5 min at 72 °C. The coding gene, *matK* was amplified with the primers, matK_3F_KIM_f (5'-CGTACAGTACTTTTGTGT TTACGAG-3') and matK_1R_KIM_r (5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3'), which were described by Jeanson et al. (2011). The amplification was conducted as 35 cycles of denaturation 94 °C for 1 min 15 s, annealing 50–52 °C for 2 min, extension 72 °C for 2 min 15 s, and final extension 72 °C for 5 min.

After the PCR amplification, the products of the reaction were purified with exonuclease I (TaKaRa Bio Inc.) and thermosensitive alkaline phosphatase (TSAP; Promega, Madison, Wisconsin, USA). Direct sequencing of purified PCR products were performed using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) using with primers on the automated sequencer (ABI prism 3130 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) or I sent purified PCR products to Eurofins Genomics Inc. (Tokyo, Japan) for genome sequencing. The nucleotide sequences were edited and assembled using Geneious software version 10.0.8 (Biomatters, Auckland, New Zealand). The present sequences were deposited to the DNA Data Bank of Japan (DDBJ)/The European Molecular Biology Laboratory (EMBL)/GenBank.

For phylogenetic analysis, samples representing a single haplotype were treated as a single operational taxonomic unit (OTU). The haplotypes obtained in this study were aligned with the sequences of *L. aequinoctialis* and the other close species, including outgroup taxa, analysed in the previous study (Bog et al. 2020a; Borisjuk et al. 2015; Kim et al. 2017; Les et al. 2002; Neubig et al. unpublished; Tang et al. 2014, 2015; Wang et al. 2010; Xu et al. 2015) using ClustalW based on software MEGA-X version 10.0.5 (Kumar

et al. 2018) for each locus. The two data matrix of 421 and 581 characters representing 13 and 12 OTUs from five species were subjected to maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) analyses by MEGA-X. Insertions/deletions in the alignment were treated as missing data. Tamura Three-Parameter (Tamura, 2011) model was suggested as the best model by model test in MEGA, and selected for ML, NJ tree construction. One of the heuristic searches (branch-swapping algorithm), Subtree-Pruning-Regrafting (SPR) topology (Nei and Kumar 2000) was used for MP tree construction. All possible subtrees from main tree were filtered and reput to all possible locations. The robustness of lineages was tested using bootstrap analysis (Felsenstein 1985) with 1,000 replications. A concatenated analysis of the two cpDNA regions was performed same as above, but only two species, *Lemna minuta* Kunth and *Lemna valdiviana* Phil. were used as outgroup.

To support the genetic relationships between all haplotypes, the Neighbor-net network tree was constructed by SplitsTree version 5.0.21 (Huson and Bryant 2018) with the same data set based on default options (Bryant and Moulton 2004; Dress and Huson 2004; Hamming 1950).

2.3 RESULT

2.3.1 Diversity of sexual reproduction

Among 13 strains of *L. aequinoctialis* complex, 11 strains excepting for two (NGY140, 178) were successfully induced flower by our Benzoic acid-adding treatment. I could observe *L. aequinoctialis* complex (Fig. 2.1a) has two typical phenomena in their sexual organ development. The first was protogyny, the pistil firstly reached out and matured (Fig. 2.1b). Even though when the pistil completely matured and droplet for pollination was on the stigma, the stamens were still in the budding pouch surrounded by spathe as primordia formation. At that time, there were two young anthers, but the filaments were not developed yet. Or else, one stamen started growing and coming out to outside of frond. However, the pistil usually started withering when the stamen started to come outside of frond.

The other was homogamy. The flowers matured pistil and stamen(s) at the same time. In almost all homogamous flowers, the one stamen started to emerge first, and then the pistil followed. They grew almost together, and the anther opened. I could often observe

the healthy stigma under the opened anther (Fig. 2.1c). When the firstly grown stamen fully ripened and open anther, the second stamen emerged. Moreover, some flower had been pollinated by self. Besides, the floral organs of both groups wilted in 24 hours.

I could observe nine to 82 flowers from each strain (Fig. 2.2). Among 39 flowers from NGY14, 37 flowers matured pistil and stamens together, and two flowers matured pistil earlier than stamens. In NGY33, I could observe 25 flowers, all flowers were homogamous. In NGY59, 37 flowers matured pistil and stamen together among 38 flowers, I could observe one protogynous flower. NGY122 bloomed the largest number of flowers, I could observe 79 flowers matured pistil earlier than stamens except to three homogamous flowers. In NGY123, all 21 flowers were protogynous. NGY128 had the smallest flower number among 11 strains. Eight flowers were protogynous, one flower was homogamous. In NGY142, 31 flowers developed pistil and anther together, but two flowers developed pistil in advance. In NGY180, 196, 302, gynoecium of 61, 27, 23 flowers matured earlier than androecium, on the other hand, androecium and gynoecium of one, eight, two flowers matured together, respectively. At last, all 73 flowers of *L. aequionctialis* no. 6746 were homogamous.

Based on the number of fronds which has specific physiological trait from each strain I could sort the strains out into two groups, A (NGY 122, 123, 128, 180, 196, 302) and B (NGY 14, 33, 59, 142, Landolt's *L. aequinoctialis* no. 6746), whose over 70 % of fronds mature pistil earlier than stamens or mature pistil and stamen(s) at same time. The rate of protogyny in group A was 74.19−100 %, and the rate of homogamy in group B was 93.94−100 % (Fig. 2.2, Table S2.3).

2.3.2 Confirmation of self-fertility

During seven days flower monitoring, I observed 234 and 208 flowers from group A and B, respectively. Some naturally self-pollinated flowers successfully fruited. The top of ripen fruit spontaneously bursted, one seed was dropped from one fruit. I found three fronds bearing fruit in strain NGY122 (group A). The fronds had enlarged ovary, and they seemed like normal fruits. However, they withered without bearing seed set. Eventually, I could not find any seed from group A despite the abundant flowers (Table 2.2).

On the other hand, group B fruited with mature seeds. The rate was 19.18−100 %,

and 63.34 % in average. The Landolt's no. 6746 showed the second highest flower number in 11 strains, but lowest fruit and seed number. The fruiting rate was 19.18 %. Except for the strain, I could get seeds from over 50 % of fronds among flowered fronds (Table 2.2).

2.3.3 Morphometric analysis

I observed and measured quantitative, qualitative morphological characters of totally 310 flowering fronds from 12 strains (Table S2.2). The flowers were induced by eight hours of light in NGY14, 33, 53, 59, 66, 140 or Benzoic acid in NGY 14, 33, 59, 122, 123, 128, 142, 180, 196, and Landolt's *L. aequinoctialis* no. 6746. The characters were compared in two groups which were distinguished by physiological traits. In 23 quantitative morphological character measurement, ranges of most characters overlapped and no differences between two groups, but in four characters (frond thickness, root cap diameter, anther length and diameter), the two groups differed (Fig. 2.3a, b, g, h, Table S2.4). The frond thickness dimensions of group A and B were measured in average 0.63 ± 0.09 mm and 0.57 ± 0.12 mm ($P < 0.0001$, Welch's test). Root cap diameter average were 0.19 ± 0.02 mm and 0.17 ± 0.03 mm ($P = 0.0002$, Welch's test). The mean anther length were 0.18 ± 0.03 mm and 0.14 ± 0.02 mm ($P < 0.0001$, Welch's test), and diameter were 0.15 ± 0.02 mm and 0.12 ± 0.02 mm ($P < 0.0001$, Welch's test).

In the qualitative character, root shape, 53.27 % of fronds of group A had straight roots, 44.86 % had wavy roots. The most plants of group B had straight roots (91.30 %). The number of fronds having spiral root was only two and one in group A and B, respectively (Fig. 2.4a). The two groups had significantly different root shape ratio ($P < 0.0001$, Pearson test).

Group A had three types (acute, obtuse, and intermediate of two types) of root tip shape. The acute root tip accounted for the largest proportion (56.31 %), intermediate and obtuse root tip shape followed (35.92, 7.77 %) in group A. On the other hand, almost all fronds of group B had acute root tip (98.48 %) and only one frond had intermediate root tip (Fig. 2.4b). Statistically significant evidence also supported the difference in two groups ($P < 0.0001$, Pearson test).

In the PCA, the two groups could be distinguished along PC axis 1 (Fig. 2.5a). The ranges of PC values along axis 1 were −0.567 to 3.676 for group A and −4.147 to 1.834

for group B. When the PC scores were averaged for each strain, the trend was clearly different between the two groups (Fig. 2.5b). Morphological relationships were not well resolved in PC2 and PC3.

The first three principal components were responsible for 69.4 % of the value. PC1 accounted for 30.1 % of total variance, which was based on spathe length, spathe diameter, ovary length, stigma width, filament diameter, and anther length. PC2, accounting for 24.1 % of total variance, was contributed by characters of spathe length, spathe diameter, stigma width, ovary length, filament diameter, and anther length (Table S2.5).

2.3.4 Molecular analyses

In *atpF–atpH* IGS, 14 haplotypes were discovered (463−686 bp) from 153 plants of *L. aequinoctialis* complex samples collected in Brundi, China, India, Japan, Korea, and US (California). In total, there were two insertions and/or deletions and one to 27 substitutions among 14 haplotypes (Table 2.3). Among them, the haplotype A13 from Japan and Korea accounted for 47.1 % (n = 72). The haplotype A3 from two lakes in China occupied the next (33.3 %; n = 51). The phylogenetic trees (ML, MP, NJ) showed two clades in *L. aequinoctialis* complex (Fig. 2.6a). The haplotype A1–5, 7–9, 11 and 12 were included in clade α, and A6, 10, 13, and 14 were included in clade β. The two clades were supported by bootstrap values (BV) 43–52 %.

In *matK* region, seven haplotypes were detected (626–853 bp) from 128 *L. aequinoctialis* complex samples in Brazil, China, Japan, Korea, and US (California). These haplotypes were identified by one to 17 substitution sites (Table 2.4). The haplotype M1 (48.4 %; n = 62) was collected from two lakes in China and Japan, and it took dominance in all haplotypes. Besides, the haplotype M6 (46.1 %; n = 59) which got the second dominance was founded only in Japan. The phylogenetic tree also showed two clades in *matK* region, M1–3, and 7 from Brazil, China, Japan, Thailand, and US (California) were in clade A, M4–6 from Japan, Korea, and US (California, Texas) were in clade B (Fig. 2.6b). The BVs of two clades were all 99 %.

I could detect 10 haplotypes in the two loci, *atpF–atpH* IGS and *matK*, concatenated sequences (1071 bp) from 125 plants of *L. aequinoctialis* complex from China, Japan, Korea, Thailand, and US (Table S2.1). The phylogenic trees showed the two monophyletic lineages in 10 haplotypes (Table 5, Fig. S2). The *atpF–atpH* IGS (A) and

matK (M) haplotype combination, Type 1–6, 9 were included to clade 1, and Type 7, 8, and 10 were included to clade 2. There were no major differences in topology with former two trees based on two of each locus (Fig. 2.6a, b), but two clades in *L. aequinoctialis* complex were more highly supported (BV > 89 % and 79 %). The neighbor net network tree also supported the topology of phylogenetic trees (Fig. 6, S2), it showed two groups in *L. aequinoctialis* complex (Fig. S2.3). Among them, Type 1 and 2 possesed in Japanese plants were phylogenetically close to Type 3. The haplotypes were closely related in each clade group. Moreover, the two groups A and B distinguished by physiological, morphological analyses belonged to molecularly divided two clades 1 and 2, respectively.

2.3.5 Haplotype distribution

The distribution map was made based on the haplotypes of Japan and Korea's *L. aequinoctialis* complex (Fig. 2.8). In Japan, three haplotypes (Type 1, 2, 10) distributed. Among them, Type10 was widely distributed in Japan, on the other hand, the distributions of Type 1 and Type2 were limited to the southern part of Japan. In the case of Korea, the haplotypes were more varied than in Japan. I could verify that there are not only Type 10 and A1, which distribute to Japan, but also A8, A9, A14 exist in Korea.

2.4 DISCUSSION

2.4.1 The physiological difference

Most Lemnoidae species are known as protogynous plants (Landolt 1957, 1986; Les 2020). Among them, especially, annual *Lemna* species (section *Alatae*) were recorded as adichogamy, self-compatible, and frequently self-pollinated species (Les 2020). Meanwhile, Beppu et al. (1985) recognized two physiological traits in Japanese *L. aequinoctialis* complex as follows: 1) protogyny and self-incompatibility and 2) adichogamy and self-compatibility. They described the characters as diagnoses of *L. aequinoctialis* and *L. aoukikusa*, respectively.

I also could find both traits not only in Japan but also in Korea, Thailand, and US. During my monitoring of flower organs' maturing timing, I observed pistil and stamen(s) of some fronds in both groups mature at the same time (Fig. 2.1c, 2.2). In this situation, without continuous (every 12 hours) monitoring, it was difficult to judge which organ grew in advance. Landolt (1986) mentioned he has never seen protogyny in *L.*

aequinoctialis. I think that it is because of the difficulty of inducing flower in *L. aequinoctialis sensu Beppu et al.* (1985). Beppu et al. (1985) mentioned that *L. aequinoctialis* doesn't flower without Salicylic acid addition. At that time, they cultivated them in greenhouse to get flowers. I also faced this kind of situation. I could not induce flower from group A using photoperiodic control, but could induce flower using Benzoic acid. Moreover, during our field trip, I sometimes could find flowers under the natural condition in summer (locality of NGY180, 184, 196, 199 in clade A, Fig. 2.8a) like as Beppu et al. (1985) did. However, it was difficult to observe the protogyny in the field because of their tiny size, rapid withering, and they often seem to be adichogamy (Fig. 2.8).

Under Benzoic acid added condition, it was sometimes difficult to get seeds from some strains. At the beginning of observation stage, the low fruiting rate of Group A seemed to be due to self sterility resulting from differences in mature timing of the flower organs. Hence, I tried to pollinate artificially in each strain, but I also could not get any mature seeds (Lee, unpublished data). In strain NGY122, although I observed three fruits, they did not successfully mature (Table 2.2). These results indicate that the plants of physiological group A have self-incompatibility. Landolt (1957) has mentioned the possibility of self-fertile and self-sterile in *Lemna perpusilla* Torrey which is one of the closely related species with *L. aequinoctialis*. He discovered matured fruit set from strain no. 6746 which belonged to physiological group B and phylogenetical clade 2 in this study (Fig. 2.1, 2, 6, S2.3, and Table 2.2, S2.3) and strain, no. 7001. At that time he identified strains no. 6612 (clade 1 in this study) and no. 6746 as *L. perpusilla*, which are now treated as *L. aequinoctialis* by Crawford et al. (2001) and Landolt (1980). Landolt (1957)'s insight about *L. perpusilla* also may be including the floral physiological traits of *L. aequinoctialis*.

Beppu (1981) conducted artificial pollination experiments in and between the two strains of S type. The different seed ripening rate under four conditions, he concluded that S type is self-incompatible. Beppu (1981)'s S type may be physiological group A. More detail pollination studies are needed for self-incompatibility of *L. aequinoctialis* complex. The self-incompatibility and quite low seed set ratio of group A and type S, and the self-compatibility of group B including strain no. 6746 and seed production ability may be related to their overwintering styles and life cycles. Landolt (1957) had mentioned their

overwintering ability, strain no. 6746, which was identified as *L. perpusilla* at that time, could not survive in winter, and *L. perpusilla* could survive as seed during the dry season. On the other hand, Beppu (1981) pointed out that the plants of S type overwinter as fronds in Kyoto, and suggested the possibility that S type in Okinawa where is more southern area than Kyoto in Japan also would be alive in winter as evergreen. I could confirm NGY122 and 123 (group A) were living as frond on January in Okinawa (Table 2.1).

Here, I detected the various physiological mechanisms in *L. aequinoctialis* complex. Similar situations have been reported by several scientists that different physiological properties in close species (Abdallah et al. 2019; Ashton and Berlyn 1994; Kusaba et al. 2001; Miri and Bubar 1966; Quero et al. 2006; Schmid 1984; Ye et al. 2003). Self-sterility may have caused genetic variation and diversity, and boost the flourishing of angiosperm after the Cretaceous Period (Whitehouse 1950). It is known as one of the important mechanisms in evolution and specification (Ferrer and Good 2012). The different overwintering type between two groups might be influenced by their different pollination mechanisms, and it also affected their geographical distribution.

2.4.2 The Morphology of L. aequinoctialis complex

This research indicated that the physiologically distinct two groups A and B had different trends in morphological characters. The physiological traits were conspecific in group A with *L. aequinoctialis sensu Beppu et al.* (1985) and group B with *L. aoukikusa*. Beppu et al. (1985) mentioned that the frond, pistil, and anther of *L. aequinoctialis* is thicker, longer, and wider than of *L. aoukikusa*. This research also showed the different trends in these characters between two physiological groups (Fig. 2.5). However, while they argued the style diameter of *L. aequinoctialis sensu Beppu et al.* (1985) was wider than *L. aoukikusa* (0.12–0.15 and 0.09–0.11 mm) and the stamen length of *L. aequinoctialis sensu Beppu et al.* (1985) were not much different with *L. aoukikusa* (0.64–1.07 and 0.64–1.21 mm), the size of former character was same in two physiological groups (Fig. 2.3d, Table S2.4), the later character showed longer size in group A (0.60 ± 0.13 mm) than group B (0.52 ± 0.14 mm) (Table S2.4).

The nomenclators of *L. aoukikusa* treated the root and root tip shapes as diagnoses (Beppu et al. 1985). Although I could not clearly divide two physiological groups based on these characters, groups A and B showed different trends (Fig. 2.4). And I also found

different trend in the root cap diameter between the groups (Fig. 2.3b). Root morphology is probable that taxonomically candidate character for identification of these groups. Landolt (1986, 1998) argued that the root cap of the subfamily Lemnoidae never regenerates unlike in land plants, and their root cap size can not be used taxonomically due to its wide range of variation. However, it is possible that there were several species mixed in what he considered to be a species.

The rib number on seed coat, seed shape, and fruit direction are taxonomically important in sect. *Alatae* (Beppu et al. 1985; Kandeler and Hügel 1974; Landolt 1986). I could not compare the fruit and seed characters in this study because of the difficulty in collecting them in group A. The characters need to be carefully observed and compared in the future study.

The overlaps and small gap between the two groups in various characters show that they are very closely related. According to Landolt's Lemnoideae geographic distribution pattern and the sample list in 1957 and 1986, at that time, he did not seem to have observed a living Japanese *L. aequinoctialis* complex. The frond thickness, which showed significant difference between two groups in *L. aequinoctialis* complex in this study, is difficult to observe/measure based on herbarium specimen materials. Even though Landolt (1986) asserted that *L. aoukikusa* is in the range of variation at world level of *L. aequinoctialis*, the discovery of differences in not only vegetative but also sexual morphological characters between two groups cultured under the same condition suggests that the independence of group B as a species-level from group A.

2.4.3 Molecule analysis

The *atpF−atpH* IGS or *matK* is one of the most commonly used cpDNA loci in phylogenetic studies of the subfamily Lemnoidae because of their high PCR amplification success rate, or suitability for species identification. Many phylogenetic research based on the *atpF−atpH* IGS have indicated the possibility that *L. aequinoctialis* is monophyletic (Borisjuk et al. 2015; Kim et al. 2017; Tang et al. 2015; Wang et al. 2010; Xu et al. 2015).

To understand the genetic relationship between physiologically and morphologically divided two groups, I added four close species, *L. minuta*, *L. perpusilla*, *Lemna tenera* Kurz, and *L. valdiviana* as outgroup for phylogenetic analysis. In the both phylogenetic

trees based on two loci, *L. perpusilla* which belongs to sect. *Alatae* were grouped with *L. aequinoctialis* complex, the others were placed as outgroup. However, there were sometimes discordance in sequences from same sample (*L. perpusilla* 8539 and *L. tenera* 9020, 9024) by different researchers (Bog et al. unpublished; Borisjuk et al. 2015). Moreover, the sequence of *L. perpusilla* 8539 (MG775397) was identical with of *L. aequinoctialis* 7126 (GU454217). Because the strain *L. aequinoctialis* 6612, 6746, 7126 and *L. perpusilla* 8539, 8612 have been identified based on morphology (and/or physiology) and the allozyme-based molecule data supported their classification (Crawford et al. 2001), their identification may be not wrong. It is possible that some sequences of *L. perpusilla* and *L. tenera* are wrong, probably due to errors in the experimental process. Since I could not determine which sequence was incorrect, I included both in the phylogenetic tree.

Meanwhile, Borisjuk et al. (2015) conducted BLAST analyses using *atpF−atpH* IGS and *psbI−psbH*, and supported the superiority of the former one for *L. aequinoctialis* identification. At that time, they added one sample of *L. aoukikusa* which was collected from a small pond in the botanical garden of Hokkaido University to their study. They offered BLASTN result of Japan sample with three *L. aequinoctialis*, 6612 (clade α), 7126 (clade β), and 6746 (clade β, group B). They showed five to eight of mismatching number and 98.26−98.91 % of matching rate between the four samples in *atpF−atpH* region, and concluded its noncoding DNA sequence is identical to which of *L. aequinoctialis*. Based on the result, they reidentified the sample as *L. aequinoctialis*. However, unfortunately, I could not judge the Hokkaido's *L. aoukikusa* in detail only by the mismatching number. Considering the result that there were three to eight substitutions (when insert/deletions were deleted) between clade α and β (Table 2.3), I can not exclude the possibility that the Hokkaido's plant is *L. aoukikusa*. As such, it may cause problem to simply identify species only by the substitution number or matching rate in DNA sequence.

The haplotype distribution (Fig. 2.7) indicates different distribution pattern in two clades. Comparing with the distribution map in Beppu et al. (1985), clade 1's distribution corresponded to of *L. aequinoctialis sensu* Beppu et al. (1985), clade 2's distribution agreed with of *L. aoukikusa*. It supports the possibility that the two clades represent the two *Lemna* species.

2.5 CONCLUSION

In this study, I induced flower, conducted various studies to detect the semi-cryptic species *L. aoukikusa* from *L. aequinoctialis s.l.* The two groups in *L. aequinoctialis* complex based on physiological, morphological difference reflected genetic distinction, and supported their independent taxonomical status. In addition, I could detect *L. aoukikusa* (Fig. 2.8b), which was treated as kind of variation of *L. aequinoctialis* only in Japan by Landolt (1986), distributes not only to Japan but also to Korea, and US (California). Contrasting in floral character which has been used in angiosperms classification for a long time, supported their taxonomical separation. Moreover, their different root cap diameter size, which has been considered not good to use to distinguish *Lemna* species, was newly discovered in this study, revealing the potential of root cap to be used more simply for taxon identification.

Most aquatic plants are heavily influenced by their habitat environment in their morphology, ecology, growth (Eckert et al. 2016; Li et al. 2020). Duckweed is one of the representative aquatic plants, it is known for having various morphology in vegetative parts. It is risky to recognize the taxonomical boundary in the genus *Lemna* only based on one type of analysis. Careful morphological, ecological, physiological, and molecular studies should be conducted in fusion.

I conducted this study using cpDNA to discover the semi-cryptic species in preliminary. To investigate their evolution relationship the nuclear-based molecular analysis is need in future study. Meanwhile, I could not recognize *L. aoukikusa* subsp. *hokurikuensis* in this study. The cytological study and observation of overwintering style are needed for taxonomical treat of the subspecies.

CHAPTER 3

Genetic diversity and population structure of *L. trisulca*: Are they clonally originated from a single plant in Japan?

3.1 INTRODUCTION

The various propagation and dispersal vectors of aquatic plants have attracted the attention of many scientists for more than a century (Darwin 1859; De Vlaming and Proctor 1968; Ridley 1930; Santamaría 2002). The aquatic plants have undergone through several evolutionary and adaptive processes to survive in their main habitat, various and extreme aquatic environments. As the result, they became able to propagate vegetatively, and furthermore, generally have a greater ability to reproduce themselves asexually (Philbrick and Les 1996, Wan et al. 2016).

The vegetative propagation allows aquatic macrophytes to increase their amount greatly in their population and help to disperse themselves otherwhere (Santamaría 2002). For example, *Myriophyllum spicatum* L. and *Elodea canadiensis* Michx. can disperse by their fragment (Nichols and Shaw 1986). It has also investigated that 80 % of *Eichornia crassipes* in Peru, which propagate in both stolons and seeds, originated from a single plant (Zhang et al. 2010). Meanwhile, seeds or plant fragments of *Najas marina* L. and *Potamogeton pectinatus* L. are eaten by birds or stuck on bird's body, and disperse to other area (Stuckey 1985; Mader et al. 1998). Les (2020) suggested that *Lemna minor* L. have a potential to disperse to 100 km or up to 1,000 km under ideal flight conditions. Thus, aquatic macrophytes may rely on the water flow and animals to spread themselves whether sexually or asexually. Understanding the macrophytes' reproduction and dispersal mechanism can be effective not limited to aquatic plants but can further affect the understanding of the ecosystem. However, research on the aquatic plants' dispersion is mainly conducted by casual observation (De Vlaming and Proctor 1968) or experimental simulation (Charalambidou and Santamaría 2002) for few decades, and fathoming their dispersion through molecular analysis has been rarely conducted.

The representative aquatic plants, duckweed, *Lemna trisulca* L. is one of the most unique species in the genus *Lemna* L. Unlike the other ca. 12 *Lemna* taxa (Beppu et al. 1985; Bog et al. 2020b) which are freely floating on the water surface, *L. trisulca* is the only species which is submerging and floating under the water surface. The obviously lot of cohering fronds, long branched chains, oblong-lanceolate frond, denticulate frond margin indicate the specialty of the species. *L. trisulca* widely distributes to temperate climate area in the world (Landolt 1986). They float to the water surface only when their flowering season. The ration of flowering was reported to be very low at 1.5 %, and the fruit-set ratio was also only 0.4 % (Landolt 1986).

Their morphological variation has also been reported by Landolt (1986). He argued that his five living collections from Australia never developed root and had only one vein on the frond under his cultural condition. And he could not find any fruits from Australia specimens. At that time, he mentioned that kind plants cannot be treated as a distinct species from *L. trisulca*.

The same situation is also known in Japan. Japanese *L. trisulca* was protected as an endangered species [Red list of Japanese Ministry of the Environment 2020; https://www.env.go.jp/press/files/jp/114457.pdf (Date accessed: 20 February 2023)]. It partly distributes to Hokkaido to Middle and North part of Honsyu [Kadono 1994; Search system of Japanese red data; http://jpnrdb.com/search.php?mode=map&q=06050276046 (Date accessed: 5 January 2023)]. Since it was firstly reported in Japan by Matsumura (1884), the flowers and fruits have never been reported (Kadono 1994, 2014). Considering that cosmopolitanization of most Lemnoideae (Araceae) species, their flower rarity, small and light body there are possibilities that they would have low genetic diversity or their high clonality.

In chapter 3 of the present study, I used Multiplexed ISSR Genotyping by Sequencing (hereafter MIG-seq; Suyama and Matsuki 2015) to inspect genetic diversity and genetic structure of Japanese *L. trisulca*. MIG-seq, a PCR-based NGS method, is known as suitable and convenient method for clone identification (Suyama and Matsuki 2015). In this chapter, I had three main objectives: (1) confirmation Japanese *L. trisulca*, whose sexual organs have never been found, are clonally originated from a single plant. (2) to elucidate the relationship between some morphological characters and genetic structure, (3) consider whether *L. trisulca*, a submerged plant, would propagate in the

same way as other free-floating species in the genus, which are known to mainly spread through vegetative propagation.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials collection

I collected 31 plants of *L. trisulca* from seven populations, which were from Iturup Island (IT), Esashi City (KC) and Akkeshi City (KN) in Hokkaido Prefecture, Nikko City (TC) in Tochigi Prefecture, Sunto City (SO) in Shizuoka Prefecture, Kitasaku City (KS) and Nagano City (NG) in Nagano Prefecture, located in shallow stream from mountain, lake, or river in Japan. Two pieces were collected from one plant in each of the two populations in Nagano Prefecture, and finally 35 species of *L. trisulca* were analyzed. All samples except for Iturup Island sample were collected from May in 2019 to September in 2020. The voucher specimens (Table 3.1) were stored in the Niigata University Herbarium (NGU) and Hokkaido University Herbarium (SAP).

3.2.2 DNA extraction and DNA barcoding

The DNA extraction were mainly conducted based on cetyltrimethylammonium bromide method (Milligan 1992). Two coding genes (*matK* and *rbcL*) and one noncoding spacer, *atpF–atpH*, which were proposed as DNA barcoding loci by Wang et al. (2010) were amplified. The noncoding gene was amplified by the same methods in Chapter 2, the others, *matK* and *rbcL* were amplified with the primers recommended by Jeanson et al. (2011) and Wang et al. (2010). The polymerase chain reaction (PCR) of *matK* was conducted as 35 cycles of denaturation 94 °C for 1 min 15 s, annealing 50–52 °C for 2 min, extension 72 °C for 2 min 15 s, and final extension 72 °C for 5 min. The *rbcL* was amplified as 95 °C for 4 min, 35 cycles of denaturation 94 °C for 30 s, 55 °C for 1 min, extension 72 °C for 1 min, final extension 72 °C for 10 min.

After the PCR amplification, the products of the reaction were purified with exonuclease I (TaKaRa Bio Inc., Tokyo, Japan) and thermosensitive alkaline phosphatase (TSAP; Promega, Madison, Wisconsin, USA). Direct sequencing of purified PCR products was performed using BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) using with primers on the automated sequencer (ABI prism 3130 Genetic Analyzer, Applied Biosystems) or I sent purified

PCR products to Eurofins Genomics Inc. (Tokyo, Japan) for genome sequencing. The nucleotide sequences were edited and assembled using Geneious software version 10.0.8 (Biomatters, Auckland, New Zealand). The present sequences were deposited to the DNA Data Bank of Japan (DDBJ)/The European Molecular Biology Laboratory (EMBL)/GenBank. To verify the genetic relationship between Japanese and abroad *L. trisulca*, I compared our sequences with the available sequences in the NCBI database.

3.2.3 Library construction in MIG-seq Analysis

The MIG-seq analysis was conducted based on the method of Suyama and Matsuki (2015). There are mainly two steps of polymerase chain reactions (PCR) and sequencing. The first PCR was conducted with eight types of MIG-seq primer set for ISSR region amplification: $(ACT)_4TG$, $(CTA)_4TG$, $(TTG)_4AC$, $(GTT)_4CC$, $(GTT)_4TC$, $(GTG)_4AC$, $(GT)_6TC$, $(TG)_6AC$. I mixed the 1 µl of template DNA, 0.2 µM of each 16 primers, 3.5 µl of 2 × Multiplex Buffer Mix (Multiplex PCR Assay Kit Ver. 2, Takara Bio Inc. Kusatsu, Japan), 0.035 µl of Multiplex PCR Enzyme Mix (Multiplex PCR Assay Kit Ver.2, Takara Bio Inc.). The final volume of mixture was set as 7 µl with water. PCR was performed with Thermal Cycler (TP650 Standard, TaKaRa Bio Inc.) under 1 min of initial activation at 94 °C, 25 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 1 min, 72 °C for 1 min, and 10 min of final incubation at 72 °C. I preliminary checked the PCR amplification using electrophoresis.

The 50^{-1} of diluted first PCR products were used in the second PCR. I mixed 3 µl of diluted first PCR product, 0.375 U PrimeSTAR GXL DNA Polymerase (Takara Bio Inc.), 3 µl of 5 × PrimeSTAR GXL Buffer (Takara Bio Inc.), 200 µM dNTP, and each 0.2 µM of second PCR primers (Suyama and Matsuki 2015). The PCR was performed under 12 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 15 s, and extension at 68 °C for 30 s. 300–800 bp of fragments in the purified library were collected by Pippin Prep DNA size selection system (Sage Science, Beverly, MA, USA).

The reads were input into Stacks v.1.47 (Catchen et al. 2011) for SNP detection. I used ‘ustacks’ option, the arrangements of the same regions were summarized, compared, organized, and combined. The option ‘population’ was conducted in Stacks program under the conditions as follows: at least 11 % of a locus shared within a population (r = 0.11), a minimum number of populations in a locus is 1 (p = 1). To select only first SNP

in locus ‘write_single_snp’ option was selected, minimum of minor allele frequency is 0.01 (min_maf = 0.01), maximum observed heterozygosity is 0.6 (max_obs_het = 0.6).

The loci were filtered by using the software TASSEL ver.5.2.77 (Bradbury et al. 2007). The filtering conditions were gradually checked and selected. The loci whose missing individual rate were over 50 %, the individuals whose missing locus rate were over 60 %. Finally, the minimum allele frequency under 0.01 were deleted.

3.2.4 Clonality determination

The genetic distance pair matrixes between individuals were constructed using TASSEL ver.5.2.77 (Table S1). To check the clonality of Japanese *L. trisulca*, I calculated the genetic distance under the three conditions. First is ‘within individual’. I calculated the genetic distance within clone. Three pieces from two each population, KS and NG built six pairs of genetic distance matrix. Second is ‘among individuals in same population’, I selected the genetic distance pair from each population where at least two plants were collected. Third is ‘between population’. I calculated the average of the genetic distance pair between populations. Based on these data, the box plot was constructed using software RStudio ver.2022.02.1. The three types of genetic distance were compared and statistically confirmed with analysis of variance (ANOVA) and T-test (Student’s test, Welch’s test).

3.2.5 Data analysis and morphology observations

The genetic diversity of each population of *L. trisulca* was determined based on number of different alleles (*Na*), number of effective alleles (*Ne*), number of private alleles, expected heterozygosity (*He*), observed heterozygosity (*Ho*), unbiased expected heterozygosity (*uHe*), inbreeding coefficient (F_{IS}) which were calculated using GenAlEx v.6.5 (Peakall and Smouse 2012).

The Bayesian cluster analyses were conducted using software STRUCTURE v.2.3.4 (Pritchard et al. 2000). The ancestral lineage *K* were set 1 to 10 with 20 replications in each *K* value, with 100,000 of burn-in and 100,000 Markov Chain Monte Carlo (MCMC) iteration. The most appropriate *K* value was estimated based on both log probability [LnP (*K*)], and Δ*K* from STRUCTURE HARVESTER v.0.6.94 (Earl and VonHoldt 2012).

With the same data set converted to GENEPOP format by software PGDspider

v.2.1.1.5 (Lischer and Excoffier 2012), principal coordinate analysis (PCoA) was conducted using GenAlEx v.6.5 to visualize the genetic relationship of each *L. trisulca* individual. With the genetic distance calculated from same data set, Neighbor-Net phylogenetic network was constructed using SplitsTree5 v.5.0.21 (Huson and Bryant 2006) based on Neighbor Net method and Splits Network Algorithm method (Bryant and Moulton 2004; Dress and Huson 2004).

To elucidate the correspond between genetic group and morphological trait, I investigated the root presence/absence and the number of frond's vein which Landolt (1986) focused on. The characters were observed based on herbarium specimens. Depending on the number of plant on a specimen sheet, at least one to at most 10 plants were observed per a population.

3.3 RESULTS

3.3.1 DNA sequencing and alignment

DNA sequences of three accessions of a representative sample from each population were amplified. The alignment length was 452–510 bp in *matK*, 570–628 bp in *atpF–atpH* IGS, 390–419 bp in *rbcL*. In these three loci, I couldn't find any variations in my Japanese *L. trisulca* samples. However, in comparison with abroad *L. trisulca*, I could find few different haplotypes in *matK* and *atpF–atpH* IGS.

In *matK* gene, I could compare my sequences with 10 sequences from Australia, Canada, Myanmar, UK, US. Among them, Canada (GU454167), UK (JN895273), and US (GU454168) sample's sequences showed one mismatch with ours under 410 bp of alignment length. In the noncoding spacer, *atpF–atpH*, mine were identical with the strain UTCC399 (GU454238), but there were four mismatches with Landolt's two strains, 7579 (GU454236) and 8137 (GU454237) under the 563 bp of alignment length. However, in *rbcL* region, all mine and 15 sequences from NCBI showed only one haplotype.

3.3.2 Clonality determination

In *L. trisulca*, I finally got 381 SNPs from 35 samples including six pieces of two plants from two populations in Nagano Prefecture. The mean (min–max) genotyping rate of all samples was 73.4 % (58.5–83.5 %). The genetic distance pair within individual (WI), among individuals in same population (AI), between populations (BP) was 0.103 ±

0.008 (0.089–0.113), 0.107 ± 0.014 (0.083–0.149), 0.249 ± 0.079 (0.134–0.359), respectively (Fig. 1). The genetic difference in three conditions were significantly different ($p < 0.0001$; ANOVA). In comparison of two conditions, WI and AI were statistically not different ($p = 0.8070$; Student's test and Welch's test), but AI and BP, WI and BP were significantly different ($p < 0.0001$; Student's test and Welch's test).

3.3.3 Genetic diversity and clustering analysis based on SNPs data

The mean values of different alleles number (*Na*) and effective alleles number (*Ne*) was 1.114 and 1.054, respectively. The mean values of observed heterozygosity (H_O) and expected heterozygosity (*He*) were 0.167 (0.087–0.223) and 0.101 (0.043–0.144), respectively. The F_{IS} values were negative in all populations (Table 2).

The STRUCTURE analysis showed the divided genetic clusters of *L. trisulca* in Japan (Fig. 2, 3, 4). The ΔK value was highest at $K = 2$ (Fig. S1a), the each ΔK value of $K = 2$ to $K = 9$ was 260.92, 0.07, 0.11, 0.08, 0.30, 0.06, 1.04, and 0.41, respectively. The log-likelihood value LnP (*K*) drastically increased between $K = 1$ to $K = 2$, and gradually increased $K = 2$ to $K = 6$ (Fig. S1b). The log likelihood of 20 runs were the most similar and stable at $K = 2$, and the genetic clusters were separated to cluster I, Hokkaido (KC, KN), Tochigi (TC), Shizuoka (SO) Prefectures, and Iturup Island and cluster II, Nagano Prefecture (KS, NG). Therefore, $K = 2$ was determined as the highest hierarchy level in the STRUCTURE analysis whereas there were relatively variable runs at $K = 3$. At $K = 2$, the *He* value of cluster I (0.205) was lower than of cluster II (0.162) and the *F* value of cluster I (0.479) was higher than of cluster II (0.583) (Fig. 3).

The 35 samples from seven populations of *L. trisulca* were evaluated by PCoA (Fig. 5). 22 samples from Nagano Prefecture (KS, NG) and the other 13 samples were separated to two groups by axis 1, which indicates 19.16 % of total variation.

3.3.4 Morphology observation

The representative morphological characters which Landolt (1986) focused on were observed. Three populations (IT, KC, KN) from Hokkaido had root (Table 3). Two (IT, KC) of them had both one or three veins, the other (KN) had only three veins. However, I could not find root from any Honshu populations (TC, SO, KS, NG). 10 plants from NG population had one vein, the other three Honshu populations had one or three veins.

3.4 DISCUSSION

According to the first aim of this study, I calculated and investigated the genetic distance under three conditions based on Suyama and Matsuki (2015)'s recommendation. The SNP loci based MIG-seq analysis let me to deliberate the clonality of *L. trisulca* in Japan. The fact that the genetic distance range of WI and AI were not significantly different suggests that plants in each population originated from one plant. However, the significantly wide range of genetic distance in BP implies that each population of Japanese *L. trisulca* originated from different individuals. In other words, *L. trisulca* usually preserves its population by asexual propagation, but each population may be generated by plant fragment or seed dispersal from other population.

In the present study, I also could get genetic structure of *L. trisulca* based on SNP loci. Although three loci chloroplast-based DNA could not detect genetic difference in the species, genome-wide SNPs detected two lineages of *L. trisulca* in Japan by a combination of STRUCTURE, PCoA, Neighbor-net network (Fig. 3.2, 3.5, 3.6). Considering that highly limited gene flow between populations and the low *He* value of two clusters (Fig. 3.1, 3.2), they may have been established by the influx of different populations (individuals), not by isolation. The long-distance dispersal of *L. trisulca*'s leaf fragment is limited to its short drying period and slow reproduction speed unlike other floating *Lemna* species (Keddy 1976). Although both the sexual and asexual propagule of aquatic plants are known to exhibit significant dispersive capabilities within hydrologically connected water bodies, it has already been reported by several scientists that asexual reproduction is important for short-distance and local spread and sexual propagation is useful for regional and long-distance spread (Clausen et al. 2002; Santamaría 2002). The present genetic distance-based results support the previous researchers' arguments.

Meanwhile, two genetic clusters did not show the typical morphological difference (Table 3.3). Both clusters had two types of root and vein number. Even within an individual, the number of veins was sometimes various. However, root character was different by locality. All Hokkaido plants had root but I never found root from Honshu plants. Although I only checked the morphology base on the dry specimen this time, it is necessary to check carefully by continuous cultivation whether these morphological features are kind of phenotype plasticity or not.

CHAPTER 4

New insight of *L. japonica*'s hybridization event in Japanese sect. *Lemna* with special reference to physiological and morphological characteristic

4.1 INTRODUCTION

The section *Lemna* is the most taxonomically problematic section in the subgenus Lemnoideae (Landolt 1986). There are seven species without *Lemna trisulca* [*L. gibba* L., *L. disperma* Hegelm., *L. minor* L., *L. japonica* Landolt, *L. obscura* (Austin) Daubs, *L. turionifera* Landolt, *L. symmeter* Giuga] in the section in whole world. In one of the smallest angiosperms, the reduced organ, extremely small sized plant, rarity of flower makes it difficult to identify. Furthermore, due to frequent hybridization occurrence in the section (Landolt 1986, Braglia et al. 2021b), the forms and characteristics of species more diverse. Sect. *Lemna* species identification mainly has been relying on vegetative end ecological characteristics, the situations cause confusion in their classification and taxonomy.

In Japan, there are three native species in sect. *Lemna*, *L. minor*, *L. japonica*, *L. turionifera* except for one exotic species, *L. gibba*. Among them, many researchers have argued *L. japonica* is a hybrid species between *L. minor* and *L. turionifera* based on their morphology, molecular trait (Braglia et al. 2021b, Hirahaya and Kadono 1995, Landolt 1986, Wang et al. 2010). However, their taxonomical identification is still problematic, their sexual characteristics have not been sufficiently reported, it caused an understanding of the relationship between the hybrid species (*L. japonica*) and its putative parental species (*L. minor* and *L. turionifera*) not sufficient. The questions, when *L. japonica* hybridized, whether Japanese *L. japonica* hybridized in Japan, why *L. japonica*'s fruit has never been reported, what difference do the three species make in floral traits, are still unresolved. Therefore, this study aimed to elucidate the nature of the hybridization event

and following hybrid speciation in Japanese sect. *Lemna*, with finding additional physiological and morphological traits to distinguish them.

4.2 MATERIALS AND METHODS

4.2.1 Plant materials collection

From May in 2019 to September in 2020 from field trip and specimen survey of Korean herbaria in 2019, 88 plants of *L. japonica*, *L. minor*, and *L. turionifera* were collected from 51 localities (18 Prefectures in Japan and four Provinces in Korea) (Table 4.1). One to six plants from each population were collected, five plants with three clone (six plants in total) were collected from population J4 (Joetsu-shi, Niigata Prefecture). The plants were usually collected from Lake, pond, or often from rice field. Korea samples were detached from specimens of Korea National Arboretum (KH).

I ordered the type strain of *L. japonica*, no. 7182 (Fukuoka, Japan) from Rutgers Duckweed Stock Cooperative (RDSC, Rutgers, US) which administers many Landolt's duckweed axenic collection. The plant was quarantined on May 31, 2022, by Agriculture Inspectors in Connecticut, US, and Japan quarantine inspector.

4.2.2 Establishment of axenic strain and culture

Method of axenic culture and the cultivation condition were same as in Chapter 2.

4.2.3 DNA extraction and DNA barcoding

Methods of DNA extraction and DNA barcoding were same as in Chapter 2, but I only sequenced non-coding spacer, *atpF–atpH H* in this chapter.

4.2.4 Library construction in MIG-seq Analysis

The main method of Library construction was same as in chapter 3. The loci were filtered by using the software TASSEL ver.5.2.77 (Bradbury et al. 2007). The filtering conditions were gradually checked and selected during analysis. The loci whose missing individual rate were over 33 %, the individuals whose missing locus rate were over 75 %. The minimum allele frequency under 0.01 were deleted.

4.2.5 Data analysis, clustering analysis, and phylogenetic tree construction

The genetic diversity of each population was determined based on same method of chapter 3.

The Bayesian cluster analyses were conducted on same method of chapter 3. However, when most appropriate *K* value was estimated, suspicious 12 runs which had too high values were deleted (Table S4.1). The principal coordinate analysis (PCoA) and phylogenetic network analysis (Neighbor-Net) were also conducted on same method of chapter 3.

To construct phylogenetic tree, the most genetically close species *L. trisulca* was selected as outgroup based on previous studies (Borisjuk et al. 2015; Tippery et al. 2015). The SNPs data set which includes *L. trisulca* was inferred the maximum likelihood phylogeny using RAxML 8.2.12 (Stamatakis 2014). I used GTRGAMMA model and conducted 1,000 replicates of bootstrap.

4.2.6 Flower induction

To observe the flower development and morphology of the three species, I tried to induce the flower with chemical substance. Main method of flower induction was same as in Chapter 2, but the 10 μM Benzoic acid was replaced by 30 μM Salicylic acid in this chapter. I added Salicylic acid to 11 strains of Japanese sect. *Lemna* (Table 4.2).

4.2.7 Flower development observation

Totally 213 plants from 10 strains (Table 4.2) were monitored every 24 hours for seven days under dissecting microscope. Detailed monitoring method was same as in chapter 2.

4.2.8 Pollen number and pollen viability estimation

To prevent pollen lose I only picked anther just opened flowers which were fixed on agar-semisolid medium. The one stamen was moved to agar-semisolid Hutner medium (1 % sucrose, 0.7 % agar) in 90 × 20 mm sized petri dish. The two anthers attached at the top of filament were opened on the medium by injection needles under dissecting microscope. The anthers from some strains, which did not open after a few days, were opened by injection needles. The pollen grains were spread within a circle of ca. 0.8

centimeters in diameter. After spreading pollen grains on the surface of medium the perti dish was closed and sealed with parafilm, and then kept under 25 ℃, 40 ± 3 μmol m^{-2} s^{-1} of continuous light from both LED and fluorescent light (Terukuni Denki, Tokyo, Japan) in incubator (LH-240N, Nippon medical & Chemical instruments, Osaka, Japan) for 24 hours. All pollen number were counted as germinated or not. Finally, pollen number from 64 plants, pollen viability from 57 plants were estimated (Table 4.2).

4.2.9 Morphological analysis

The all plants were cultivated on liquid medium. To measure morphological characters, five to ten flowering ramets from each strain were collected. Two qualitative and 23 quantitative morphological characters which have been treated as important characters for sect. *Lemna* species identification were investigated. Totally 55 plants from eight strains were observed. Box plots were constructed to visualize the morphology measurements.

The longest root was selected to observe and measure. Flowers within 3 days of pistil development were collected and measured for flowers in a state in which the pistil has not withered yet. For the sizes of the anther and its filament, the longer one of two stamens was measured. The measured characters were statistically tested by Chi-Square test, Mann-Whitney Wilcoxon test, or T-test (Student's test, Welch's test) to reveal the significance of (sub)clusters from SNP loci based on clustering analyses.

To conduct principal component analysis (PCA), I selected the characters having high correlation for each of the principal factors, and then, removed characters which were highly correlated (which had the value over 0.7) to each other in covariability (Table S4.2). Finally, six (frond number, frond thickness, ovary diameter, stamen length, root cap length) characters were selected.

4.2.10 Genetic diversity consideration of Japanese *L. japonica* based on clonality determination

The genetic distance pair matrixes between individuals were constructed using TASSEL ver.5.2.77 (Table S4.3). To check the clonality of the hybrid species, *L. japonica*, I calculated the genetic distance under the three conditions same as chapter 3.

4.2.11 Hybridization time estimation with Approximate Bayesian Computation

I selected Approximate Bayesian computation procedure (ABC) to estimate the origin of *L. japonica* and the speciation time using the software package DIYABC v2.1 (Cornuet et al. 2014). According to the clustering analysis, I selected one and two samples from subcluster a, b in cluster A (Pop 1), and three samples were selected from cluster B (Pop 2) and C (Pop 3), respectively. When I select samples the genetic distance pair matrixes between individuals constructed by TASSEL ver.5.2.77 were carefully confirmed, samples with a low probability of originating from a clone was selected by using 'Taxa Filter' option in TASSEL. All nine samples (NGY86, 96, 117, 151-st1, 158-11, 191-1, 200-2, 204-st2, 207) were collected from different locality. The vcf file was converted to EIGENSOFT format using PGDspider v.2.1.1.5 (Lischer and Excoffier 2012). In addition to minor allele frequencies < 0.01, which was set up when constructing library, the complete missing loci were deleted. The file was finally converted to file extension format as '.snp'.

At the beginning of analysis, totally 14 scenarios were tested. And then, highly supported six scenarios were tested in full-scale (Table S4.4). For scenario 1, the common ancestor, the Pop1 and Pop2 are originated from common ancestor, Pop3 is diverged from Pop2. For scenario 2 and 3, two populations (Pop1 and Pop3 or Pop1 and Pop2) are originated from common ancestor, and the hybrid event occurs between two parental populations. For scenario 4 and 5, Pop1 and Pop3 or Pop1 and Pop2 are diverged at t2 and t1 (timescale measured in generation) by cladogenesis from Pop2 or Pop3. Finally, in scenario 6, the three Pop are diverged from common ancestor at same time.

For parameter settings, I selected summary statistics (Genic diversities, Fst distances, Nei's distances) for each population and each population pair to compare the observed and simulated data comparing. Totally 10 summary statistics (proportion of zero values, mean of non-zero values, variance of non-zero values) were employed. I assessed 10^7 simulations for each scenario.

To estimate posterior probabilities of each scenario 1,000 data sets was selected. A logistic regression was conducted to each simulation data, and 1 % of simulated data indicating the most similarity to the observed data. PCA was constructed with 'model checking' option in DIYABC to visually assess the observed data set position with the simulated data sets.

4.3 RESULTS

4.3.1 DNA sequencing and alignment

DNA sequences of *atpF–atpH* IGS of a representative sample from each population were successfully amplified. Three haplotypes (M, J, T) were discovered from 52 plants in Japanese native species in section *Lemna*. The alignment length was 449–619 bp in M, 485–614 bp in J, 560–574 bp in T. Each haplotype pair had 1, 16, 15 substitutions in J–M, J–T, T–M combination, respectively (Table 4.3). The haplotype M was identical with *L. minor* strain no. 9417 (GU454231, Germany), J was identical with *L. japonica*'s type strain, no. 7182 (GU454225, Japan), T was identical with *L. turionifera* strain no. 7683 (MG775404, Korea) of Landolt's collection.

4.3.2 Genetic diversity and clustering analysis based on SNPs data

The mean values of different alleles number (*Na*) and effective alleles number (*Ne*) was 0.782 and 0.779, respectively. The mean values of observed heterozygosity (H_O) and expected heterozygosity (*He*) were 0.058 (0.003–0.141) and 0.030 (0.001–0.072), respectively. The F_{IS} values were negative in all populations (Table 4.4).

The STRUCTURE analysis showed the divided genetic clusters of section *Lemna* in Japan (Figure 4.1). The ΔK value was highest at $K = 2$ (Figure S4.1a), the each ΔK value of $K = 2$ to $K = 9$ was 5769.13, 616.99, 0.79, 0.21, 0.74, 0.82, 0.48, and 1.41, respectively (Table S4.1). The log-likelihood value LnP (*K*) drastically increased between $K = 1$ to $K = 2$, and gradually increased $K = 2$ to $K = 4$ (Figure S4.1b). So, I attached the structure topology of $K = 2$ to $K = 5$ (Figure 4.1). The log likelihood of 15–20 runs were the most similar and stable at $K = 2$, and the genetic clusters were obviously separated to cluster A which had M type of cpDNA, cluster B which had J type of cpDNA, and cluster C which shows mixture of cluster A and B, had T type of cpDNA except for P51 (Table 4.1). From $K = 3$, Cluster A divided to two subclusters a and b, stably. The subcluster a corresponded to P1 to P6, subcluster b corresponded to P7 to P24. But in P2, both subcluster a and b were detected (Figure 4.1).

The 88 samples from 51 populations of Japanese section *Lemna* were evaluated by PCoA (Figure 4.2). I retained two PCs, accounting for 47.20 % of total variance. All plants in cluster A were clearly separated from cluster B and C by principal coordinate axis 1, which indicates 31.91 % of total variation. The cluster B and C were distributed along

axis 2 which indicates 15.29 % of total variation. The Neighbor-Net result also revealed three clusters similar as result of STRUCTURE analysis, but there was one more subcluster in subcluster b (Figure 4.3).

4.3.3 Physiological characteristics: based on flower induction

The flowers of all 10 strains of Japanese section *Lemna* strains were successfully induced by 30 μM Salicylic acid-adding treatment. All flowers were protogynous, and there was water droplet on stigma. There were two types of flowers; 1) stamen normally develop (stamen matures after pistil mature, but their maturing timing is not much different) and dispersing pollen grains by naturally opened anther (Figure 4.4c, 4.5c), and 2) stamen does not develop until pistil withers, and the anther never opens (Figure 4.6e, f, g). In detail, those stamens slowly grew, and they were pushed out and dropped as the daughter frond grew (Figure 4.6h). The flower of subcluster a (in cluster A) and cluster C were same as type 1. The flower type of subcluster b (in cluster A), cluster B, and *L. japonica* type strain 7182 were 2.

4.3.4 Morphology observation and morphometric analysis

I observed and measured two qualitative and 23 quantitative morphological characters of totally 55 flowering fronds from eight strains (Table 4.2). Considering the results of clustering analyses and physiology observation, firstly, the flower spathe color could divide three groups in Japanese native section *Lemna*. The cluster A had transparent spathe, cluster B, C, and *L. japonica* type strain 7182 had pigmented spathe (Figure 4.4b, 4.5b, 4.6e, f). Because the subcluster b did not mature stamen during pistil alive, I excluded it, and only subcluster a, cluster B and C were observed and compared in this study.

Pigment on upper frond near apex were observed from totally 42 plants. 90.9 % of subcluster a did not have pigment near apex of upper frond, in cluster B with *L. japonica* 7182 56.25 % had pigment, 43.75 % did not have pigment, 100 % of cluster C plants had pigment (Figure 4.7a). Three groups were significantly different in the character ($P < 0.0001$, Pearson test). 23.07 % of subcluster a had same height of papillae on frond's midvein, but 76.92 % had different height of papillae. 40 % of cluster B with *L. japonica* 7182 had same height of papillae, but 60 % had different. 80 % of cluster C had same

heighted papillae but 20 % had different height of papillae (Figure 4.7b). Three groups were significantly different in the character ($P = 0.0100$, Pearson test).

The 23 quantitative morphological character measurement were presented on Table 4.5. Ranges of most characters overlapped and no differences between two or three (sub)clusters, but few characters showed the most significant difference (root diameter, root cap diameter, stigma width) of the three groups (Table 4.5, Figure 4.8).

The frond number of three (sub)clusters were counted in average 5.67 ± 2.52, 4.09 ± 1.15, and 2.40 ± 0.63 ($P = 0.0001$, ANOVA and Wilcoxon test). Frond thickness was 0.64 ± 0.12 mm, 0.90 ± 0.11 mm and 0.63 ± 0.18 mm ($P < 0.0001$, Welch's test). Root diameter was 0.12 ± 0.03 mm, 0.17 ± 0.01 mm and 0.14 ± 0.02 mm ($P < 0.0001$, Welch's test), root cap diameter was 0.18 ± 0.02 mm, 0.23 ± 0.03 mm and 0.19 ± 0.03 mm ($P = 0.0001$, ANOVA and Wilcoxon test). Stigma width was 0.19 ± 0.04 mm, 0.23 ± 0.03 mm and 0.15 ± 0.03 mm ($P = 0.0001$, Welch's test).

In the PCA, cluster B and *L. japonica* 7182 was distinguished from (sub)cluster a and C along PC axis 1 (Fig. 4.9). The ranges of PC values along axis 1 were −1.994 to 0.087 for subcluster a, 0.534 to 2.733 for cluster B with *L. japonica* 7182, and −3.499 to 0.588 for cluster C. Cluster B with *L. japonica* 7182and C were divided along PC axis 2. The ranges of PC values along axis 2 were −0.176 to 4.096 for subcluster a, −1.874 to 0.965 for cluster B, and −1.776 to 0.208 for cluster C. Frond thickness and stigma width mostly influenced to PC1, frond number and ovary diameter were the most important characters to PC2 (Table 4.6). The three groups showed their morphological difference in PCA.

The first three principal components were responsible for 79.70 % of the value. PC1 accounted for 44.98 % of total variance, which was based on frond number, frond thickness, ovary diameter, stigma width, stamen length, root cap length. PC2, accounting for 25.49 % of total variance, was contributed by same characters of PC1 (Table 4.6).

4.3.5 Pollen number and viability of three clusters

The pollen number was 489 ± 113 (207−764) in subclade a, 444 ± 115 (279−675) in clade B with *L. japonica* 7182, 419 ± 114 (221−663) in cluster C (Figure 4.10a). In statistical test, (sub)cluster a and B (with *L. japonica* 7182), B (with *L. japonica* 7182) and C were not significantly different, but a and C were significantly different ($P = 0.1096$,

Student's test and Welch's test).

Pollen grains started germinating in about 15 minutes on contact with the agar-semisolid Hutner medium (Figure 4.11). Germinated pollen rate was 17.27 ± 8.13 (6.86−37.88) in subclade a, 0.03 ± 0.11 (0.00−0.43) in clade B, 57.35 ± 21.01 (20.36−84.52) in cluster C (Figure 4.10b). All three groups' pollen viability were significantly different ($P < 0.0001$, Student's test and Welch's test).

4.3.6 Clonality determination of cluster B

In Japanese *L. japonica*, I finally got 162 SNPs from 39 samples including four pieces of one strain which was collected from population PJ2, Joetsu-shi in Niigata Prefecture (Table 4.7; sample number 5, 8, 9, 10). The mean (min–max) genotyping rate of all samples was 71.8 % (46.2–100 %). The genetic distance pair within individual (WI), among individuals in same population (AI), between populations (BP) was 0.072 ± 0.014 (0.059–0.089), 0.083 ± 0.035 (0.037–0.185), 0.110 ± 0.037 (0.060–0.257), respectively (Fig. 4.12). The genetic difference in three conditions were significantly different ($P < 0.0001$; Welch's test). In comparison of two conditions, WI and AI were statistically not different ($P = 0.4825$; Student's test), but AI and BP, WI and BP were significantly different ($P < 0.0001$, $p = 0.0115$, respectively; Student's test).

4.3.7 *L. japonica*'s hybridity verification using Approximate Bayesian Computation

After additional SNP filtering, a dataset consisting of 217 SNPs from nine plants from nine populations was used for DIYABC analysis in this study. Finally, 59,104,700 simulations for six scenarios (Figure 4.13) which were highly supported in first preliminary test conducted on 14 scenarios were performed. Among them, the posterior probability was highest in the Scenario 2 (Figure S4.2). In the model checking for scenario 2, the PCA showed that the observed data were highly similar to the simulated data sets (Figure 4.14). Parameter estimates for scenario 2 suggest that hybridization event between *L. minor* and *L. turionifera* occurred, and the original mode values of t1, t2, and t3 were 6.85×10^3 (95 % Confidence Interval: 2.10×10^3–1.31×10^4), 1.77×10^4 (95 % CI: 8.64×10^3–2.89×10^4), 3.19×10^4 (95 % CI: 1.82×10^3–3.94×10^4) generations, respectively. The original mode of the effective population sizes N1, N2, N3, and Na were 7.16×10^3 (95 % CI: 3.21×10^3–1.11×10^4), 5.80×10^3 (95 % CI: 1.47×10^3–1.28×10^4),

4.53×10^3 (95 % CI: 2.16×10^3–7.23×10^3), and 6.16×10^3 (95 % CI: 8.71×10^2–9.66×10^3), respectively.

4.3.8 Phylogenetic relationship in Japanese sect. *Lemna*

To construct the phylogenetic tree, the data set including *L. trisulca* was filtered, and 97 samples having 416 SNP loci remained. The Maximum Likelihood tree showed clear three major lineages, the lineages 1 and 2 were supported by 100 % and 98 % bootstrap value (Fig. 4.15). Most samples reflected the haplotypes but the samples which had haplotype 'T' were distinguished to two groups, one of them was grouped with haplotype 'J' sub lineage. One sample which had haplotype 'M' was grouped with lineage 1 with 32 % of bootstrap value. The lineage 2 showed its monophyly, and three each lineage was supported 21 %, 97 %, and 79 % of bootstrap value.

4.4 DISCUSSION

4.4.1 Species identification based on molecular, physiological, and morphological analyses

Although many molecular and morphological studies on section *Lemna* have been conducted for over 20 years (Les et al. 2002; Hirahaya and Kadono 1995; Volkova et al. 2023), their classification or identification is still not easy. Most studies did not include *L. japonica*'s type strain, and in cases where molecular classification was difficult, the authors suspected misidentification of *L. minor* and *L. japonica* samples which were used in their study. Recently, extensive re-identification of section *Lemna* in Landolt's collection from various parts of the world by Tubulin-Based Polymorphism (TBP) method (Braglia et al. 2021b), and the hybridization of *L. japonica* from *L. minor* and *L. turionifera* was supported, but its hybridization timing and the background were never considered.

In particular, few is known about the flowers of sect. *Lemna*, but the flowers of *L. japonica* are only known to be similar to of *L. turionifera* (Landolt, 1980) and have not been recorded in detail. It is known that *L. minor* and *L. turionifera* have difference fruit morphology, but no fruits have been found in *L. japonica*. In addition, because these three species are self-incompatible, finding (observing) fruits is more difficult than in flowers (Les 2020, Landolt 1986). Therefore, the classification and identification of *L. japonica*

would be very difficult to resolve without checking *L. japonica*'s type strain.

In this study, I was able to genetically classify the three species, and *L. japonica*' s type strain had a light pinked petal color, stamen did not develop until stigma completely wilt, and the anther(s) never opened. As a result of examining one cpDNA locus (*atpF–atpH* IGS) with the clusters which were divided based on SNP loci of Japanese sect. *Lemna* which were collected by myself and observing flowers, cluster B was same as *L. japonica*'s type strain (no. 7182) in cpDNA, flower color, and stamen development degree, so cluster B was identified as *L. japonica s.s*. Meanwhile, cluster A was divided into subcluster a and b under the condition of $K = 3$ or higher in the STRUCTURE analysis, this cluster was expected to be *L. minor* because it differed only by one SNP from *L. japonica* on cpDNA. In cluster A, all plants had transparent flower spathe, and they had no pigment. However, unlike *L. japonica* and *L. turionifera*, which generally have pigmented frond, the lower frond surface of *L. minor* has almost no pigment. Therefore, the pigmented spathe is considered to reflect the pigmented frond.

Considering that *L. minor* is described as having winged fruit, it is inferred that L. minor is a fertile species. However, as a result of observing subcluster b, all of them did not develop stamens until stigma wilt and did not open anther. Normally developing or not-developing stamens within the species has been reported in *L. gibba* (Fu et al. 2017, Landolt 1986) which is closely related taxa within the same section, and cluster A is considered to be in a similar situation. The subcluster a developed stamens and opened the anthers spontaneously, pollen grains dispersed. Thus, I judged that it is fertile, and identified it as *L. minor*. I could get one not winged fruit from strain NGY200-13 in cluster C. It was consistent with what was mentioned in the description of *L. turionifera*, I identified it as *L. turionifera*.

4.4.2 Consideration of *L. japonica*'s hybridity

The demographic history of Japanese sect. *Lemna* by Approximate Bayesian Computation most highly supported *L. japonica* was hybridized between *L. minor* and *L. turionifera*. In majority of angiosperm species, the chloroplast DNA is maternally inherited. The identical cpDNA except for one SNP in *atpF–atpH* between *L. minor* and *L. japonica* indicates the high possibility that *L. minor* is more likely to be the mother of *L. japonica* than *L. turionifera*.

It has frequently been reported that hybrid species have the intermediate characteristics of their parent species (Rieseberge 1997). Typically, *L. japonica* is known to have evenly various characteristics (turion forming ability, pigmented frond) from the putative parental species, *L. minor* and *L. turionifera*. In this study, I additionally founded some morphological characters of *L. japonica*, frond number, frond width, flower spathe color and length, ovary diameter, occupied the intermediate position between *L. minor* and *L. turionifera*. The pollen number of *L. japonica* was also moderate figure for the two putative parent species (Figure 4.10a). These kinds of intermediate characteristics may can support that *L. japonica* was hybridized between *L. minor* and *L. turionifera*.

The pollen viability of *L. turionifera* was about three times that of *L. minor*. In addition, I could observe some pollen of *L. turionifera* germinated pollen tube outside of anther even before the anther opened yet, and most pollen germinated hanging from the anther after the anther opened (Figure 4.5c). This phenomenon occurred in the sealed petri dish, and I have never detected this kind of situation in genus *Lemna* in Japan. In nature, when *L. minor* and *L. turionifera* bloom, whether at the stigma of *L. turionifera* or *L. minor*, the pollen of *L. turionifera* would be more likely to reach the ovary than pollen of *L. minor*. Furthermore, this phenomenon would have affected the combination pattern of the father and mother species in the hybridization event.

There are several factors that can be cited as candidates for causing the infertility of hybrids (Li et al. 2020), one of the representative factors is a different ploidy level of the parental species (Watkins 1932). The chromosome number of type strain of *L. japonica* was reported as 2n = 50, and which of parental species, *L. minor* and *L. turionifera* were reported as 2n = 20, 30, 40, 42, 50, 63, 126, and 2n = 36, 38, 40, 42, 50, 80, respectively (Geber 1989; Hoang et al. 2019; Landolt 1980; Urbanska et al. 1980; Wang et al. 2011). In this study I could not perform cytological analysis in Japanese sect. *Lemna* samples which I collected, and I cannot conclude that ploidy level or chromosome number is a key cause of *L. japonica*'s male-sterility. However, considering that the sect. *Lemna* is monophyletic, and their chromosome number are various, some combination of parental lineage caused sterility of *L. japonica*. A cytological analysis needs to be conducted and its relationship between the three species have to be considered carefully in future study.

4.4.3 Distributional pattern and the relationship of ecological traits, and the importance of additional morphological traits for identification

Landolt (1986) mentioned *L. japonica* can live in warmer area than *L. minor*. This insight corresponded with my result; *L. japonica* distributed Southern Japan whereas *L. minor* distributed to Northern Japan (Figure 4.16, 4.17). *L. minor* is known to be able to propagate as a seed, but not to make turions. On the other hand, the seed of *L. japonica* has never been reported, and I detected *L. japonica* is a stamen-sterile species in this study, so it seems that *L. japonica* cannot propagate sexually. In the clonality test based on comparison genetic distance under three conditions, most *L. japonica* distributes in Japan derived from clone, 1) *L. japonica* originated in many places abroad 2) actually *L. japonica* does seed propagation 3) genetic variation was occurred due to somatic cell variation. The third prediction is likely, but it was difficult to conclude on this study. It should be carefully studied in detail in future research.

L. minor, distributes in the northern part of Japan, where the temperature is relatively low (the temperatures drop below –30℃ in Hokkaido; https://www.skiing-hokkaido.com/about-hokkaido/) may overwinter as seed, and *L. japonica*, distributes in the southern part of Japan, where the temperature is relatively high, may overwinter with turion, as is the characteristic of *L. japonica* reported before.

Japan belongs to East Asia, the main distribution of *L. japonica*, and has been attracting attention as a type locality of *L. japonica*. 28 years ago, Hirahaya and Kadono (1995) conducted a study about Japanese *L. minor s.l.* (Japanese sect. *Lemna* in this study) based on allozyme, cytological, morphological analysis. The distribution map which they provided showed little different topology of mine, distributions of *L. minor* and *L. japonica* were not clearly divided as Northern and Southern Japan. The situation may be caused by not enough sampling in this study, or low quality of allozyme resolution at that time. Anyway, Hirahaya and Kadono (1995) could not distinguish *L. japonica* and *L. turionifera* based on frond's pigment. It shows that the identification based on only frond pigment is challengeable in sect. *Lemna* and indicates the need for more morphological traits for their correct identification.

CHAPTER 5

New report of two Lemnoideae species in Korean flora: *Landoltia punctata* and *Lemna turionifera*

5.1 INTRODUCTION

Lemnoideae, commonly known as duckweed, are free floating or submerged aquatic plants from the family Araceae (Iles et al 2015; Nauheimer et al 2012). The subfamily Lemnoideae includes five genera—*Landoltia* D. H. Les & D. J. Crawford, *Lemna* L., *Spirodela* Schleid., *Wolffiella* Hegelm., and *Wolffia* Horkel ex Schleid.—and consists of 40 taxa (Beppu et al 1985; Landolt 1986; Les et al 2002). Duckweed has 0–21 roots, 1–16 frond veins, and have thin or inflated, orbicular, obovate, or lanceolate fronds (Landolt 1986, 1998). Duckweeds are widely distributed across the globe, except for in polar and desert regions.

The first records of Lemnoideae in Korea were *Lemna minor* L. and *Spirodela polyrhiza* (L.) Schleid. by Nakai (1911). Chung et al (1937) also recorded these two taxa in Korea and gave them the Korean names, "*Jom-gae-gu-ri-bap*" and "*Gae-gu-ri-bap*" respectively. However, 15 years later, Nakai (1952) revised his record of *Le. minor* to *Le. paucicostata* Hegelm. Then, Park and Oh (1986) recorded three Korean species identified by Landolt: *Le. aequinoctialis* Welwitsch (*Jom-gae-gu-ri-bap*), *Le. japonica* Landolt, and *S. polyrhiza*. Although this was the first record of *Le. japonica* in Korea, Park and Oh mentioned only one locality and did not include voucher specimens or their taxonomical characteristics. In the 2000s, Lee (2006) suggested *Le. perpusilla* Torrey as the scientific name of *Jom-gae-gu-ri-bap* and treated *Le. paucicostata* as a synonym of the species. Choi (2007) recorded *Jom-gae-gu-ri-bap* as *Le. perpusilla* instead of *Le. paucicostata* with *S. polyrhiza*. Recently, Kim et al (2017) projected the possibility that Korea has over five taxa of Lemnoideae, including *La. punctata*, *Le. aequinoctialis*, *Le. minor*, *S. polyrhiza*, and *Wolffia arrhiza* (L.) Horkel ex Wimm., based on phylogenetic study.

Despite the researchers' efforts, there are still poor species recognition, Korean Lemnoideae are sometimes misidentified and reported. Based on my years of studying

duckweed species, I report new distributions of two species in Korea, *Landoltia punctata* (G. Mey.) Les & D. J. Crawford and *Lemna turionifera* Landolt, and suggest new characters, here.

5.2 MATERIALS AND METHODS

5.2.1 Plant collection, culturing in axenic conditions, and plant materials observation

From May 11 to 13, 2018, on June 7, 2019, on July 9, 2022, I collected *La. punctata* in Jejudo, and plants (NGY403) similar to *L. turionifera* on a bed of the Banbyeoncheon stream in Andong, Korea. The voucher specimens were deposited in the herbarium of Niigata University (NGU). I tried to observe plant morphological traits under axenic culture conditions to eliminate the influence of phenotypic plasticity in natural environments. The axenic culture method was same as in Chapter 2 and 4.

To identify NGY403, I observed the plant and determined its identity based on keys and descriptions provided by Landolt (1975, 1980, 1986). In addition, I compared NGY403 with few of my genetically and morphologically identified NGY collections which were collected from Korea and Japan, and which were studied in Chapter 2 and 4, (Table 5.1).

5.2.2 DNA analysis

To genetically identify Korean *La. punctata*, and NGY403, I conducted genetic analysis using the sequences of the chloroplast DNA non-coding region (*atpF–atpH*), with same methods of chapter 2.

5.2.3 Herbarium specimen survey

To examine the morphological characteristics and distribution of these species in Korea, I visited three herbaria: the herbarium of National Institute of Biological Resources (KB), the Korea National Herbarium (KH), and the Osaka Museum of Natural History (OSA). The OSA contains many Korean aquatic plant specimens, including the collections of Dr Shigeru Miki (1901–1974), who collected aquatic plants in East Asia.

5.3 RESULTS AND DISCUSSION

5.3.1 Field survey, axenic cultivation, and plant materials observation

I found two populations of *La. punctata* in Jejudo, Korea (Figure 5.1); their habitats were artificial wetlands. The first locality was in Seonheul-ri of Jeju-si, in a small shady pond surrounded by artificial stonewall. Besides the pond, there was a non-natural trail. This habitat is 50 meters from a residential area. The maximum water depth of the pond was 50 cm. The *Landoltia* plants had grown sympatrically with *Cardamine occulta* Hornem., *Carex dimorpholepis* Steud., *Ludwigia ovalis* Miq., *Maclura tricuspidata* Carrière, *Murdannia keisak* (Hassk.) Hand.-Mazz., *Persicaria lapathifolia* (L.) Delarbre, *Persicaria thunbergii* (Siebold et Zucc.) H. Gross, and *Ranunculus sceleratus* L.

At the second locality, in Hamdeok-ri of Jeju-si, I found just one individual of *La. punctata*. The irrigation pond where it was found was about 639 square meters in extent and was surrounded by stonewall. *Spirodela polyrhiza*, *Nelumbo nucifera* Gaertn., *Persicaria lapathifolia*, and *Trapa* sp. were found with *La. punctata*.

I found *L. turionifera*-like plants (Figure 5.2) in the Banbyeoncheon stream at Yongsang-dong in Andong, Korea. The collection site was ~600 m before the intersection of the Banbyeoncheon and the Nakdonggang river, the longest river in South Korea [Encyclopedia of Korean Culture; http://encykorea.aks.ac.kr/Contents/Item/E0011565 (Date accessed: 6 November 2022)]. Plants were widely spread along the streamside and the water surface was mainly covered by *Salvania natans* (L.) All. accompanied by *Nymphaea* cv., *Sparganium erectrum* L., *Spirodela polyrhiza* (L.) Schleid., and *Trapa* sp. (Figure 5.3). The frond color of the plants was purple or green on the upper side (Figure 5.2A). I did not find any flower and fruit, but I found some fronds bearing turions (Figure 5.2D). At this location, the *L. turionifera*-like plants seemed to mainly propagate vegetatively, with turions overwintering under the water. According to the results of Landolt's 1750-sheet specimen survey, the ratio of flowering specimen of *L. turionifera* was 4 %, and the ratio of specimen with fruit-set was only 1.2 % (Landolt 1986). His results are consistent with our presumption regarding the reproduction of *L. turionifera*-like plants in the stream.

After a minimum of 15 days of *in vitro* culture, I obtained measurements of the morphology and physiology of 15 ramets from strain NGY403-1 (Figure 5.2B; Table 1). The fronds were slightly gibbous, the frond length to width ratio ranged from 1.35 to 1.76.

Landolt (1986) argued that the size and position of the papule can be used to identify species in the section *Lemna*, and at the tip the papule of *L. turionifera* is not significantly larger than other papules. There were usually five papillae of about the same height along the midvein of the frond (Figure 5.2C). Unlike *L. minor*, which has a green dorsal frond surface, NGY403-1 showed a pigmented root base where it attached to the downside of the frond (Figure 5.2D). I also observed six flowering plants. I detected the pistil matures earlier than stamens (Figure 5.4C, 5.2E–F). It was consistent with the observations of *L. turionifera* by former researchers (Landolt 1986; Les 2020). All flower spathes of NGY403-1 were reddish purple colored (Figure 5.2C, 5.2E). To date, the flower color of *L. turionifera* has not yet been reported. After a month, NGY403-1 formed purplish turions (Figure 5.2G). According to Landolt (1975)'s assertion that *L. turionifera* produces turions under unfavorable environments, the formation of turions may be caused by the loss of nutrients in the medium. All observed fronds of NGY403-1 had obtuse root tips (Figure 5.2H). Therefore, I concluded that NGY403 is *L. turionifera* based on morphological traits.

5.3.2 Identification based on cpDNA sequences

The chloroplast DNA sequence (684 bp; LC511770) of the Lemnoideae plant collected from Jejudo (Seonheul-ri; collection number, NGU 8185) was completely identical with the 14 *Landoltia* sequences in DDBJ/EMBL/GenBank, namely, GU454209, GU454210, GU454212, GU454213, GU454214 (Wang et al 2010), KF726173 (Tang et al 2014), KJ630554, KJ630555 (Zhang and Xu. unpublished), KP017617, KP017622, KP017639, KP017651, KP017665 (Tang et al 2015), and AB819469 (Kittiwongwattana and Thawai unpublished). These 14 sequences are based on plants collected from south Africa, Australia, China, India, and the US. This result suggested that the Lemnoideae plant collected in Jejudo was genetically identified as *La. punctata*. Thus, the Lemnoideae plant collected in Jejudo was genetically identified as *La. punctata*.

The *atpF-atpH* IGS sequence (538 bp; LC738859) of *Lemna* sample collected from Andong (specimen number, NGU14034; strain number, NGY403-1) was found to be identical to DNA sequences of Landolt's *L. turionifera* strains (NCBI accession number MG775403, strain no. 6573; MG775404, no. 7683; MG775405, no. 9434) sourced from Russia, Korea, and the USA. Moreover, they also showed a 99.8 % match with two other

L. turionifera sequences found in China (GU454239, no. 8339) and in the Czech Republic (GU454240, no. 8760) (Table 2). The sequences of three species, *L. japonica*, *L. minor*, and *L. obscura*—which all belong to the section *Lemna*, and are genetically close to *L. turionifera* (Les et al. 2002; Tippery et al. 2015)—showed matches of 96.5 %–97.2 % to the sequences of our *Lemna* sample (Table 5.2).

Therefore, based on morphological and physiological observations of axenic cultured plants and on comparison of cpDNA sequences, I conclude that the *Lemna* plants collected from Andong were *L. turionifera*.

5.3.3 Taxonomic treatment

Landoltia punctata (G. Mey.) Les & D. J. Crawford, Novon 9: 532 (1999)

≡*Lemna punctata* G. Mey., Prim. Fl. Esseq. 262 (1818). ≡*Spirodela punctata* (G. Mey.) C. H. Thomps., Rep. (Annual) Missouri Bot. Gard. 9: 28 (1898). TYPE: Chile. Tierra del Fuego Island, Orange Harbor. Wilkes Exped. 1838; [Neotype – US, Isoneotype - GH; designated by Landolt (1986), Photo!]

=*Lemna oligorrhiza* Kurz, J. Linn. Soc., Bot. 9: 267 (1867). ≡*Spirodela oligorrhiza* (Kurz) Hegelm., Lemnac. 147 (1868). TYPE: India, West Bengal, Calcutta. 1865 [Holotype – illustration in J. Linn. Soc., Bot. 9: 267. t. 5. 1867]

Description: Roots (1 or) 2–4; penetrating small prophyllum, 8.1–31.6 mm long, 0.1–0.2 mm diameter; fronds floating on water surface, obovate to elliptic, upper surface light green to deep green, sometimes reddish edge, white minute punctate, 3–13 papillae on midvein, lower surface green or red, veins 1–4, 2.3–3.2 mm long, 1.4–2.0 mm wide, (1 or) 3–10 coherent in group; flower not seen in Korea.

Korean name: Jeom-gae-gu-ri-bap

Specimen examined: Korea. Jejudo: Jeju, 33°30’ 57” N, 126°42’26”E, Alt. ca 80 m, 12 May 2018, T. Shiga & H. J. Choi 9878 (NGU 8184); 7 Jun. 2019, T. Shiga & Y. Lee 10698 (NGU 8185); 33°31’23.4” N, 126°39’58.5” E, Alt. 34 m, 7 Jun. 2019, Y. Lee & T. Shiga 28 (NGU 8183).

Distribution and habitat: China, India, Indonesia, Japan, Korea (Jeju; Seonheul-ri, Hamdeok-ri), Malaysia, Philippines, Taiwan, Thailand, southern and eastern Asia (Landolt 1986).

Taxonomic notes: This species was first described by Meyer (1818) with specimens collected from the Essequibo River, Guyana, northern South America. However, no one could find his specimen even the contemporary duckweed researchers (Thompson 1898). After Thompson (1898) firstly made a description based on the collection collected by team of Charles Wilkes during the "United States South Pacific Exploring Expedition", Landolt (1986) designated it as a neotype.

In 21th century, However, Ward (2011) expressed doubts about this neotype. He referred to comments of Landolt (1986) that there had been no *S. punctata* reported in southern South America except for Charles Wilkes's collection collected from Orange Harbour and argued that Thompson's collections were probably mislabeled.

There have been different opinions about the identity of the collections from Orange Harbour, Chile. Researchers from North America reported this species as *S. oligorrhiza* (Kurz) Hegelm. following Hegelmaier, whereas European researchers followed Thompson in using *S. punctata* (Saegar 1934; Daubs 1962; Hartog and Plas 1970; Landolt 1986). The leading specialist of Lemnoideae, E. Landolt, commented that, due to Meyer's missing collection, the identity of *Le. punctata* G. Mey. is questionable and Meyer's description seems to match poorly developed plants of *S. intermedia* (Landolt 1986). However, Landolt additionally mentioned in a personal communication in 2012 (Wayne's Word website; https://www2.palomar.edu/users/warmstrong/PeerReview1.htm; [Date accessed: 4 December 2019]) that Meyer's description best matches *S. oligorrhiza*. He checked specimens collected from Orange Harbor during Wilkes Expedition in 1898 and they were clearly identified as what is now called "dotted duckmeat". Furthermore, he insisted that this problem cannot be completely solved, so trying to change the type would cause endless confusion.

Ward (2011) identified the basic specimen of Thompson's description as *S. intermedia*. However, *S. intermedia* is quite different from *La. punctata*. According to Meyer's (1818) description, *La. punctata* has 2–3 roots and silver-colored punctate fronds, but *S. intermedia* has 7–20 roots. Its frond size is also larger than that of *La. punctata*. Based on

these findings, we considered that the collection from “United States South Pacific Exploring Expedition” can be used as a type of *La*. *punctata*.

Lemna turionifera Landolt, Aquat. Bot. 1: 355, 1975.

Type. USA. Montana, Lincoln Co., 20 km west of Davensport, 19 vi 1953, *M*. *Hiesey s.n.* (Holotype: ZT).

Description. Annual or perennial floating herbs, Fronds ovate to elliptic, 1–4(–6) cohere in one plant, veins 1–3, 4–7(–9) similar sized papillae on midvein, 2.7–3.1 mm long, 1.9–2.2 mm wide, 1.4–1.8 times as long as wide, 0.6–0.8 mm thick, upper frond usually green, sometimes reddish purple to purplish with pigment, lower frond usually pigmented; Bisexual flower in the budding pouch in frond, protogynous, ovary 0.3–0.4 mm long, 0.2–0.3 mm diameter, style 0.2–0.3 mm long, stamen 0.4 mm long, spathe reddish colored, 0.9–1.1 mm long, 0.8–1.0 mm diameter; anther 0.2 mm long, 0.2 mm diameter. Turion very widely ovate to very widely obovate, tinged dark purple to brown, 0.97–1.29 mm long, 0.86–1.18 mm wide; root solitary, root tip obtuse, root cap 0.8–2.1 mm long, 0.2–0.3 mm diameter, root 0.8–2.3 cm long, root base dark purple–colored. No fruits and seeds had been seen in Korea.

Specimen examined. KOREA. Gyeongsangbuk-do Province, Andong, 36°33’19.2” N, 128°44’46.0” E, alt. ca 90 m, 19 vii 2022 (Y. Lee et al.) (specimen number, NGU14034; strain number, NGY403-1, 403-2, 403-3, 403-4).
JAPAN. Hokkaido Prefecture, Akkeshi-shi, 7 viii 2020, *M*. *Yamazaki* (NGU14031; NGY151c); Sunagawa-shi, 7 viii 2020, *T*. *Shiga* (NGU14032); Miyagi Pref. Higashimatsushima-shi, 17 ix 2020, *S*. *Kato* (NGU14033; NGY200-11, 200-13, 200-14); Osaka Pref., Takatsuki-shi, 12 iv 1995, *I*. *Nishikawa* (OSA183336); Shiga Pref., Shinasahi-machi, 10 v 1996, *S*. *Fujii 5113* (OSA119772); Kyoto Pref., Kyotanabe-shi, 18 v 2008, *K*. *Seto 65926* (OSA). Landolt living collection: 7427 (Tokyo, Japan).

Distribution. Canada, China, Europe (Czech Republic, Finland, Germany, Poland, Turkey, UK), India, Japan, Korea (Andong), Mexico, Mongolia, Russia, US (Braglia et al. 2021;

Halder and Venu 2012; Landolt 1986)

Remarks. (1) Morphological traits and values in the "Description" are based on observations for plants under axenic culture conditions. (2) *L. turionifera* belongs to the section *Lemna* with *L. minor*, which is known as the most difficult section to identify in the Lemnoideae (Landolt 1986). Frond color, regular papillae height, and turion formation are treated as key characters, but the difficulty of observing papillae size in the field or of determining changeable frond color based on the environment make identification difficult. The self-incompatible flower occasionally blooms and rarely fruits (Landolt 1986; Les 2020). (3) Landolt identified a *Lemna* species collected from Sosa, Kyonggi Province (Korea) by Yong No Lee as *L. japonica*. This was recorded as strain number 7683 (Landolt 1980). After 30 years, he reidentified this strain as *L. turionifera* (Bog et al. 2010). (4) We propose the Korean name of the species as Dong-a-jom-gae-gu-ri-bap (동아좀개구리밥). This name reflects the turion-forming character of the species.

Key to Korean Lemnoideae species

1. Root absent, stamen 1……*Wolffia arrhiza*

– One or more roots per frond, stamen 2……2

2. Prophyllum present

3. have both primary and secondary roots at the early frond growth stage, fronds circular to oblate, 11–17 veins on frond, 4.6–9.5 mm long, 5.2–8.6 mm wide……*Spirodela polyrhiza*

– have only primary root, fronds obovate to elliptic, 1–4 veins on frond, 2.3–3.2 mm long, 1.4–2.0 mm wide……*Landoltia punctata*

–Prophyllum absent……*Lemna*

4. Root tip acute, root sheath winged, root base always green……*Lemna aequinoctialis*

– Root tip obtuse, root sheath not winged, root base dark purple or green 5

5. Root base green, frond not gibbous, no pigment on lower surface, not turion-forming ……*Lemna minor*

– Root base usually purple-colored, frond usually gibbous, pigment on lower surface, turion-forming……*Lemna turionifera*

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Table 2.1. Collected *Lemna* strains and their information. The origin of the plants, GenBank accession number, and haplotypes were provided. Except for ** marked two sequences from Landolt strains, all sequences were generated in this study. *: Landolt collection, **: already analyzed in previous study

No.	Strain	Country	Locality	Latitude & Longitude	Collection date	Accession number		Haplotype		Voucher
						atpF-H	*matK*	*atpF-H*	*matK*	
	L. aequinoctialis complex									
1	NGY160	Japan	Aomori Prefecture	40°54'04.0"N, 140°19'22.0"E	Aug. 27 2020	LC752806	LC752984	A13	M6	Lee et al. (NGU14372)
2	NGY156	Japan	Aomori Pref.	40°52'44.0"N, 140°18'39.0"E	Aug. 26 2020	LC752807	LC752985	A13	M6	Lee et al. (NGU12806)
3	NGY159	Japan	Aomori Pref.	40°52'02.0"N, 140°17'52.0"E	Aug. 27 2020	LC752808	LC752986	A13	M6	Lee et al. (NGU14373)
4	NGY162	Japan	Aomori Pref.	39°42'27.0"N, 141°05'14.2"E	Aug. 29 2020	LC752809	LC752987	A13	M6	Lee et al. (NGU14374)
5	NGY14	Korea	Gangwon-do	38°29'33.0"N, 128°25'02.0"E	Jun. 09 2019	LC752810	LC752988	A13	M6	Lee et al. (NGU14375)
6	NGY32	Japan	Niigata Pref.	38°19'19.0"N, 138°28'16.0"E	Jun. 30 2019	LC752811	LC752989	A13	M6	Lee et al. (NGU12809)
7	NGY13	Korea	Gangwon-do	38°14'24.0"N, 128°33'39.5"E	Jun. 09 2019	LC752812	LC752990	A13	M6	Lee et al. (NGU14376)
8	NGY11	Korea	Gangwon-do	38°14'23.2"N, 128°33'57.8"E	Jun. 08 2019	LC752813	LC752991	A13	M6	Lee et al. (NGU14377)
9	NGY5	Japan	Niigata Pref.	38°05'16.4"N, 139°23'17.4"E	May 28 2019	LC752814	LC752992	A13	M6	Lee et al. (NGU14378)
10	NGY6	Japan	Niigata Pref.	38°03'41.4"N, 139°23'58.4"E	May 28 2019	LC752815	LC752993	A13	M6	Lee et al. (NGU14379)
11	NGY31	Japan	Niigata Pref.	37°55'55.0"N, 138°18'39.0"E	Jun. 29 2019	LC752816	LC752994	A13	M6	Lee et al. (NGU12808)
12	NGY202	Japan	Niigata Pref.	37°50'19.1"N, 139°14'07.4"E	Oct. 04 2020	LC752817	LC752995	A13	M6	Lee et al. (NGU14380)
13	NGY112	Japan	Niigata Pref.	37°47'54.1"N, 139°16'49.8"E	Oct. 16 2019	LC752818	LC752996	A13	M6	Shiga et Shiga (NGU12786)
14	NGY131	Japan	Niigata Pref.	37°18'43.3"N, 138°53'01.5"E	Jul.18 2020	LC752819	LC752997	A13	M6	Shiga et al. (NGU12796)
15	NGY19	Japan	Niigata Pref.	37°13'48.5"N, 138°58'07.9"E	Jun. 15 2019	LC752820	LC752998	A13	M6	Lee et al. (NGU14381)
16	NGY147	Japan	Toyama Pref.	36°53'36.3"N, 137°26'53.4"E	Jul. 28 2020	LC752821	LC752999	A13	M6	Shimono (NGU12803)
17	NGY146	Japan	Nagano Pref.	36°50'00.2"N, 138°12'11.3"E	Jul. 28 2020	LC752822	LC753000	A13	M6	Shimono (NGU12802)
18	NGY106	Japan	Tochigi Pref.	36°39'04.0"N, 140°05'43.0"E	Sep. 23 2019	LC752823	LC753001	A13	M6	Lee et al. (NGU12787)
19	NGY148	Japan	Toyama Pref.	36°35'15.1"N, 136°54'26.3"E	Jul. 28 2020	LC752824	LC753002	A13	M6	Shimono (NGU12804)
20	NGY177	Japan	Ishikawa Pref.	36°17'08.0"N, 136°17'42.0"E	Sep. 21 2020	LC752825	LC753003	A13	M6	Lee et al. (NGU12777)
21	NGY130	Japan	Gunma Pref.	36°15'47.0"N, 138°56'23.1"E	Jun. 21 2020	LC752826	LC753004	A13	M6	Naito (NGU12795)
22	NGY129	Japan	Gunma Pref.	36°15'44.2"N, 138°56'18.6"E	Jun. 21 2020	LC752827	LC753005	A13	M6	Naito (NGU12794)
23	NGY140	Korea	Gyeongsangbuk-do	36°11'52.7"N, 129°22'12.7"E	Jul.19 2020	LC752828	LC753006	A13	M6	Kim et al. (ANH-20200719-001)
24	NGY77	Japan	Fukui Pref.	35°56'18.0"N, 136°15'06.5"E	Jul. 19 2019	LC752829	LC753007	A13	M6	Shimono (NGU12789)
25	NGY143	Japan	Nagano Pref.	35°53'08.7"N, 138°18'04.7"E	Jul. 27 2020	LC752830	LC753008	A13	M6	Shimono (NGU12801)
26	NGY167	Japan	Yamanashi Pref.	35°50'05.0"N, 138°23'41.0"E	Sep. 11 2020	LC752831	LC753009	A13	M6	Lee et Naito (NGU14382)
27	NGY56	Japan	Yamanashi Pref.	35°35'07.7"N, 138°31'16.7"E	Jul. 16 2019	LC752832	LC753010	A13	M6	Nakamura (NGU12817)
28	NGY57	Japan	Yamanashi Pref.	35°35'07.7"N, 138°31'16.7"E	Jul. 16 2019	LC752833	LC753011	A13	M6	Nakamura (NGU12818)

Table 2.1. continued

No.	Strain	Country	Locality	Latitude & Longitude	Collection date	Accession number		Haplotype		Voucher
						atpF-H	*matK*	*atpF-H*	*matK*	
	L. aequinoctialis complex									
29	NGY178	Japan	Shiga Pref.	35°30'59.0"N, 136°07'23.0"E	Sep. 21 2020	LC752834	LC753012	A13	M6	Lee et al. (NGU12778)
30	NGY142	Japan	Yamanashi Pref.	35°29'27.8"N, 138°27'04.2"E	Jul. 27 2020	LC752835	LC753013	A13	M6	Shimono (NGU12800)
31	NGY192	Japan	Tottori Pref.	35°28'03.0"N, 133°56'00.0"E	Sep. 22 2020	LC752836	LC753014	A1	M1	Lee et al. (NGU14383)
32	NGY180	Japan	Shimane Pref.	35°26'06.0"N, 133°01'19.0"E	Sep. 22 2020	LC752837	LC753015	A1	M1	Lee et al. (NGU14384)
33	NGY72	Japan	Shiga Pref.	35°25'45.6"N, 136°01'46.2"E	Jul. 10 2019	LC752838	LC753016	A13	M6	Shimono (NGU127531)
34	NGY150	Japan	Gifu Pref.	35°18'39.3"N, 136°43'33.3"E	Jul. 29 2020	LC752839	LC753017	A13	M6	Shimono (NGU12805)
35	NGY41	Japan	Shiga Pref.	35°14'01.0"N, 136°17'09.0"E	Jul. 07 2019	LC752840	LC753018	A13	M6	Lee et al. (NGU12811)
36	NGY166	Japan	Shizuoka Pref.	35°10'45.0"N, 138°32'36.0"E	Sep. 11 2020	LC752841	LC753019	A13	M6	Lee et Naito (NGU12776)
37	NGY45	Japan	Shiga Pref.	35°05'14.0"N, 136°11'02.0"E	Jul. 07 2019	LC752842	LC753020	A13	M6	Lee et al. (NGU12813)
38	NGY44	Japan	Shiga Pref.	35°05'11.0"N, 136°11'04.0"E	Jul. 07 2019	LC752843	LC753021	A13	M6	Lee et al. (NGU12812)
39	NGY74	Japan	Hyogo Pref.	35°04'55.9"N, 135°16'26.0"E	Jul. 14 2019	LC752844	LC753022	A13	M6	Shimono (NGU12829)
40	NGY76	Japan	Kyoto Pref.	35°04'12.0"N, 135°47'07.2"E	Jul. 16 2019	LC752845	LC753023	A13	M6	Shimono (NGU12788)
41	NGY181	Japan	Shimane Pref.	35°01'06.0"N, 132°43'17.0"E	Sep. 23 2020	LC752846	LC753024	A13	M6	Lee et al. (NGU12779)
42	NGY75	Japan	Hyogo Pref.	35°01'03.6"N, 134°59'28.8"E	Jul. 14 2019	LC752847	LC753025	A13	M6	Shimono (NGU12830)
43	NGY51	Japan	Kyoto Pref.	35°00'58.7"N, 135°46'03.4"E	Jul. 13 2019	LC752848	LC753026	A13	M6	Lee et al. (NGU14385)
44	NGY52	Japan	Kyoto Pref.	35°00'58.7"N, 135°46'03.4"E	Jul. 13 2019	LC752849	LC753027	A13	M6	Lee et al. (NGU14386)
45	NGY53	Japan	Kyoto Pref.	35°00'58.7"N, 135°46'03.4"E	Jul. 13 2019	LC752850	LC753028	A13	M6	Lee et al. (NGU14387)
46	NGY54	Japan	Kyoto Pref.	35°00'58.7"N, 135°46'03.4"E	Jul. 13 2019	LC752851	LC753029	A13	M6	Lee et al. (NGU14388)
47	NGY73	Japan	Kyoto Pref.	34°59'55.8"N, 135°34'37.5"E	Jul. 14 2019	LC752852	LC753030	A13	M6	Shimono (NGU12828)
48	NGY199	Japan	Hyogo Pref.	34°39'01.0"N, 134°59'35.0"E	Sep. 25 2020	LC752853	LC753031	A2	M1	Lee et al. (NGU12785)
49	NGY49	Japan	Nara Pref.	34°36'55.0"N, 135°58'53.0"E	Jul. 08 2019	LC752854	LC753032	A13	M6	Lee et al. (NGU12815)
50	NGY48	Japan	Nara Pref.	34°36'52.0"N, 135°58'52.0"E	Jul. 08 2019	LC752855	LC753033	A13	M6	Lee et al. (NGU12814)
51	NGY185	Japan	Hiroshima Pref.	34°26'04.0"N, 133°16'30.0"E	Sep. 24 2020	LC752856	LC753034	A13	M6	Lee et al. (NGU12782)
52	NGY184	Japan	Hiroshima Pref.	34°25'41.0"N, 133°14'24.0"E	Sep. 24 2020	LC752857	LC753035	A1	M1	Lee et al. (NGU12781)
53	NGY196	Japan	Hiroshima Pref.	34°25'40.7"N, 133°14'22.7"E	Sep. 24 2020	LC752858	LC753036	A2	M1	Lee et al. (NGU12784)
54	NGY183	Japan	Hiroshima Pref.	34°24'56.0"N, 132°43'13.0"E	Sep. 23 2020	LC752859	LC753037	A13	M6	Lee et al. (NGU12780)
55	NGY50	Japan	Osaka Pref.	34°23'58.0"N, 135°19'22.0"E	Jul. 08 2019	LC752860	LC753038	A13	M6	Lee et al. (NGU12816)
56	NGY33	Japan	Tokushima Pref.	34°09'44.0"N, 134°33'38.0"E	Jul. 05 2019	LC752861	LC753039	A13	M6	Lee et al. (NGU12810)

Table 2.1. continued

No.	Strain	Country	Locality	Latitude & Longitude	Collection date	Accession number		Haplotype		Voucher
						atpF-H	*matK*	*atpF-H*	*matK*	
	L. aequinoctialis complex									
57	NGY60	Japan	Fukuoka Pref.	33°48'26.1"N, 130°47'57.6"E	Jul. 22 2019	LC752862	LC753040	A13	M6	Lee et Naito (NGU12820)
58	NGY61	Japan	Fukuoka Pref.	33°42'46.2"N, 130°51'23.0"E	Jul. 23 2019	LC752863	LC753041	A13	M6	Lee et Naito (NGU12821)
59	NGY59	Japan	Fukuoka Pref.	33°38'24.7"N, 131°03'01.6"E	Jul. 21 2019	LC752864	LC753042	A13	M6	Lee et Naito (NGU12819)
60	NGY36	Japan	Ehime Pref.	33°31'01.0"N, 132°59'06.0"E	Jul. 06 2019	LC752865	LC753043	A13	M6	Lee et al. (NGU14389)
61	NGY71	Japan	Kochi Pref.	33°21'44.1"N, 133°15'04.5"E	Jul. 18 2019	LC752866	LC753044	A1	M1	Maeda (NGU12827)
62	NGY128	Japan	Kochi Pref.	33°21'44.1"N, 133°15'04.5"E	May 28 2020	LC752867	LC753045	A1	M1	Maeda (NGU12793)
63	NGY70	Japan	Kochi Pref.	33°21'44.1"N, 133°15'04.1"E	Jul. 18 2019	LC752868	LC753046	A1	M1	Maeda (NGU12826)
64	NGY67	Japan	Oita Pref.	33°15'43.0"N, 131°20'54.0"E	Jul. 25 2019	LC752869	LC753047	A13	M6	Lee et Naito (NGU12824)
65	NGY64	Japan	Kumamoto Pref.	32°09'08.8"N, 130°31'02.8"E	Jul. 24 2019	LC752870	LC753048	A13	M6	Lee et Naito (NGU12822)
66	NGY66	Japan	Miyazaki Pref.	32°07'13.8"N, 131°24'23.9"E	Jul. 25 2019	LC752871	LC753049	A13	M6	Lee et al. (NGU14390)
67	NGY65	Japan	Miyazaki Pref.	32°02'59.0"N, 130°56'01.9"E	Jul. 24 2019	LC752872	LC753050	A13	M6	Lee et Naito (NGU12823)
68	NGY123	Japan	Okinawa Pref.	26°42'25.7"N, 128°08'56.0"E	Jan. 25 2020	LC752873	LC753051	A1	M1	Yamazaki et Tone (NGU12791)
69	NGY122	Japan	Okinawa Pref.	26°42'24.4"N, 128°08'56.2"E	Jan. 25 2020	LC752874	LC753052	A1	M1	Yamazaki et Tone (NGU12790)
70	NGY302	Thailand	Thoong Aruan	14°36'N, 102°11'E	unknown	LC752875	LC753053	A11	M7	Lee et al. (NGU14391)
	L. minuta									
71	9476*	UK	England	Gloster		MK516250**	LC753054			
	L. valdiviana									
72	NGY189	Japan	Hyogo Pref.	34°39'01.0"N, 134°59'35.0"E	Sep. 25 2020	LC752876	LC753055	-	-	Lee et al. (NGU14392)
73	9222*	Bolivia	La Paz			LC752877	LC753056			
74	9475*	Brazil	Manaus			MK516244**	LC753057	-	-	

Table 2.2. The number of flowering and seed-bearing individuals. All strains were fixed on the semi-solid medium and self-pollinated spontaneously. The seeds were completely matured and naturally dropped from the fruit. The seed rate is calculated based on flower and seed number.

Strain	Flowering individual	Fruiting individual	Seed number	Seed rate (%)
NGY122	82	3	0	0
NGY123	21	0	0	0
NGY128	9	0	0	0
NGY180	62	0	0	0
NGY196	31	0	0	0
NGY302	29	0	0	0
NGY14	39	23	23	58.97
NGY33	25	25	25	100
NGY59	38	20	20	52.63
NGY142	33	30	30	90.91
6746	73	14	14	19.18

Table 2.3. Variation sites of 14 haplotypes of *atpF–atpH* intergenic spacer (IGS) chloroplast DNA of *Lemna aequinoctialis* complex. The numbers indicate variable in the region.

	SNP position in *atpF-atpH* IGS																										
												1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	4
	1	3	7	8	9	9	9	9	9	9	9	1	2	4	4	4	4	4	1	3	6	6	9	3	8	8	4
Haplotype	4	6	1	4	0	1	2	3	4	5	6	5	0	2	3	4	5	6	5	7	4	5	0	9	4	9	7
A1	T	A	G	A	T	T	-	-	T	T	T	T	A	T	T	A	A	T	T	G	A	C	C	C	C	C	C
A2	T	A	G	A	T	T	T	-	T	T	T	T	A	-	-	-	-	-	T	G	A	C	C	C	C	C	C
A3	T	A	G	A	T	T	-	-	T	T	T	T	A	-	-	-	-	-	T	G	A	C	C	C	C	C	C
A4	T	A	C	A	T	T	-	-	T	T	T	T	A	-	-	-	-	-	T	G	A	C	C	C	C	C	C
A5	T	A	G	A	T	T	-	-	T	T	T	T	A	-	-	-	-	-	T	G	A	C	C	T	T	G	A
A6	T	A	G	G	T	T	T	T	T	T	T	A	G	-	-	-	-	-	T	G	A	C	A	C	C	C	C
A7	T	A	G	G	T	T	T	T	T	T	T	A	A	-	-	-	-	-	T	G	A	G	C	C	C	C	C
A8	T	G	G	A	T	T	-	-	T	T	T	T	A	-	-	-	-	-	T	G	A	C	C	C	C	C	C
A9	T	A	G	A	T	T	-	-	T	T	T	T	A	-	-	-	-	-	G	G	A	C	C	C	C	C	C
A10	T	A	G	G	T	T	-	-	T	T	T	A	G	-	-	-	-	-	T	A	A	C	A	C	C	C	C
A11	T	A	G	G	T	T	-	-	-	T	T	A	A	-	-	-	-	-	T	G	A	G	C	C	C	C	C
A12	T	A	G	G	T	-	-	-	-	T	T	A	A	-	-	-	-	-	T	G	A	G	C	C	C	C	C
A13	G	A	G	G	-	-	-	-	-	-	-	A	G	-	-	-	-	-	T	G	A	C	A	C	C	C	C
A14	G	A	G	G	-	-	-	-	-	-	-	A	G	-	-	-	-	-	T	G	-	C	A	C	C	C	C

Table 2.4. Variation sites of 7 haplotypes of *matK* chloroplast DNA of *Lemna aequinoctialis* complex. The numbers indicate variable in the region.

	SNP position of *matK*																
		1	1	1	1	2	2	2	2	2	3	3	4	4	4	4	5
	2	0	4	5	7	2	3	3	6	9	2	7	0	0	5	9	6
Haplotype	1	3	1	3	0	7	3	4	4	1	6	5	3	8	9	0	4
M1	A	A	C	C	A	G	G	G	A	C	C	C	G	C	C	G	T
M2	A	A	C	C	A	A	G	A	A	C	C	C	G	C	C	G	T
M3	A	A	C	C	A	G	G	G	A	C	C	C	C	A	C	C	T
M4	A	G	C	A	G	G	G	G	A	C	G	C	G	C	G	G	T
M5	A	A	C	A	A	G	G	G	C	C	G	A	G	C	G	G	A
M6	G	A	T	A	A	G	G	G	A	C	G	G	G	C	G	G	T
M7	A	A	C	C	A	G	C	G	A	A	C	C	G	A	C	C	T

Table 2.5. The 10 haplotypes concatenated two chloroplast DNA of *Lemna aequinoctialis* complex collected from China, Japan, Korea, Thailand, and US. "n" indicates the number of sequences. The A7, 8, 9, 12,14, and M3, 5 were not included in this table because they could not concatenate due to the lack of *atpF–atpH* IGS or *matK* sequences in GenBank.

Concatenate haplotype	Haplotype		n
	atpF-H	*matK*	
1	A1	M1	8
2	A2	M1	2
3	A3	M1	50
4	A3	M2	1
5	A4	M1	1
6	A5	M1	1
7	A6	M4	1
8	A10	M4	1
9	A11	M7	1
10	A13	M6	59

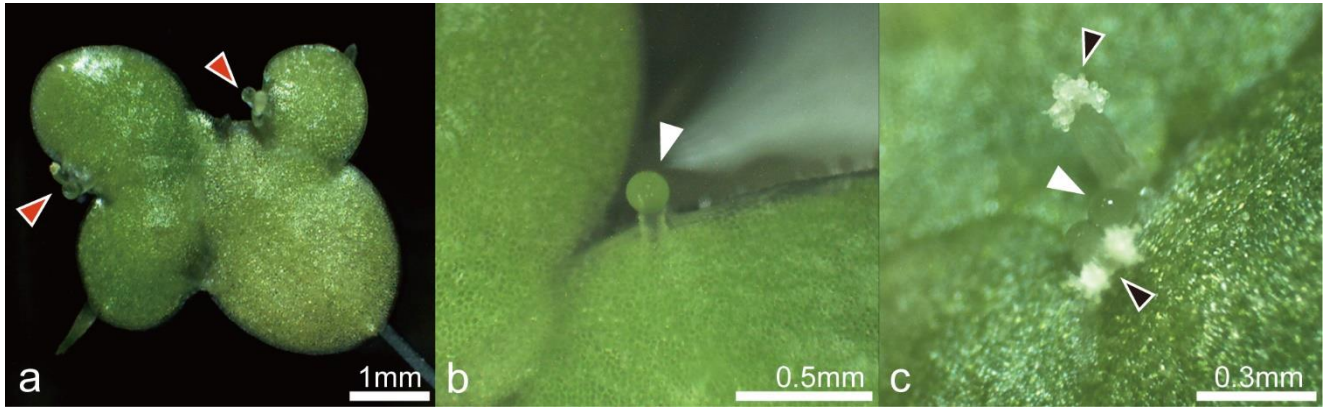


Fig. 2.1. Flowers of *Lemna aequinoctialis* complex a A whole plant of flowering *L. aequinoctialis* complex (NGY13), the red arrows indicate flowers. b A pistil (white arrow) matured alone (NGY122). A droplet on the stigma indicates the maturity of the pistil. c Both stamens (black arrows) and a pistil (white arrow) mature together. The anthers fully matured and opened; the droplet is on the stigma (NGY33).

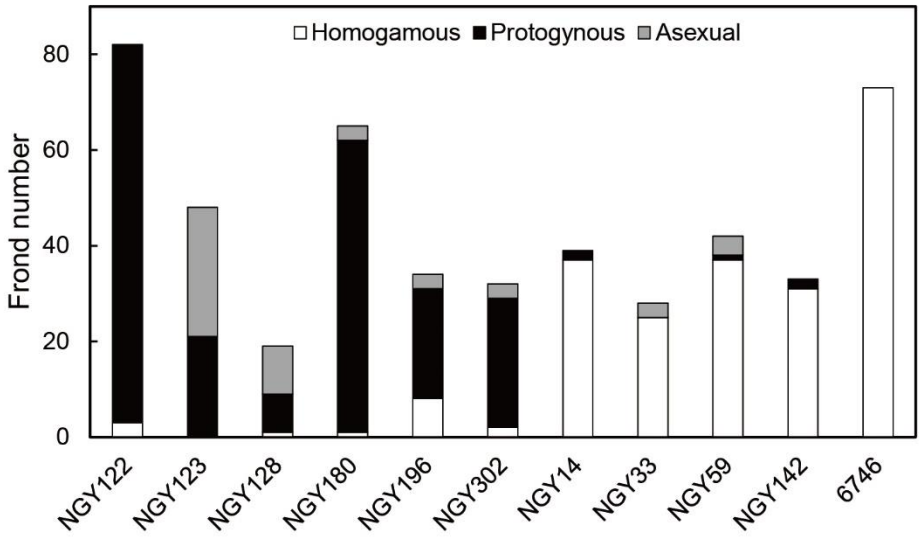


Fig. 2.2. Flower organ's mature timing of each strain. The flowers were observed every 12 hours for seven days. The black color indicates the protogynous flower number whose pistil matures earlier than stamen(s). The white color indicates the homogamous flower number whose stamen(s) and pistil mature together in the same moment. The gray color indicates not bloomed fronds number.

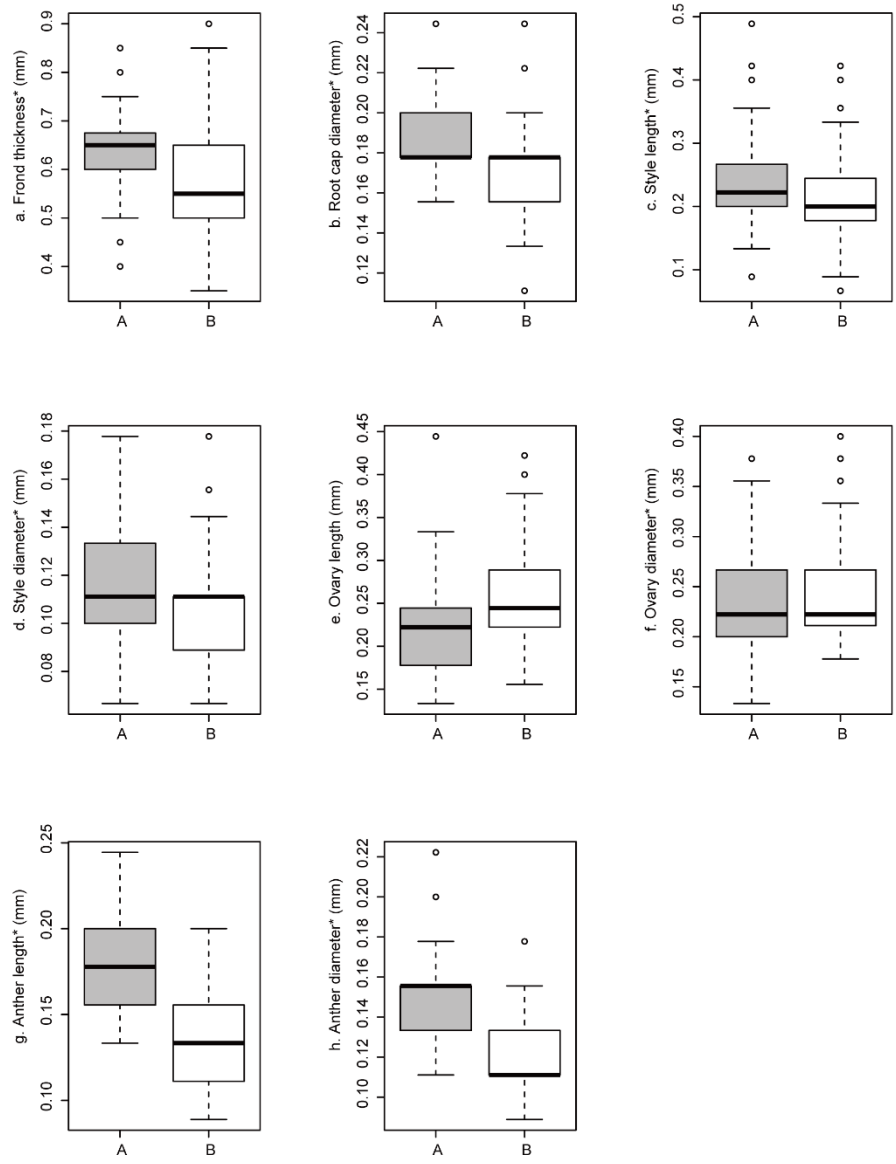


Fig. 2.3. Eight morphological characters of *L. aequinoctialis* complex. a frond thickness, b root cap diameter, c style length, d style diameter, e ovary length, f ovary diameter, g anther length, h anther diameter. Asterisked (*) characters (frond thickness, root cap diameter, style length, style diameter, ovary diameter, anther length, anther diameter) are the characters having different trend that description of *L. aequinoctialis* and *L. aoukikusa*. For each box plot, top bar indicates maximum value, top end of the box indicates third quartile, the middle bar indicates median value, and the circles indicate possible outliers.

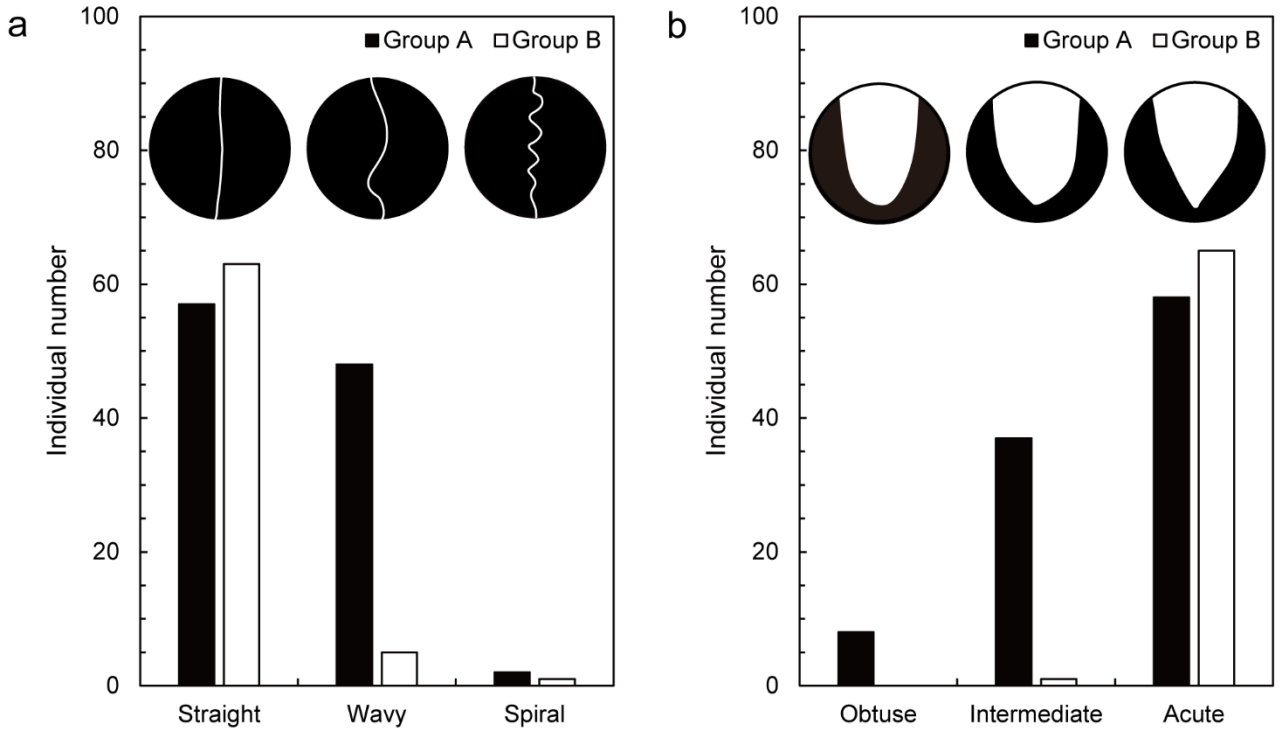


Fig. 2.4. Root morphology of *L. aequinoctialis* complex. a The individual number of which has a straight, wavy, and spiral root shape on two groups. b The individual number of which has an obtuse, intermediate, and acute root tip on two groups.

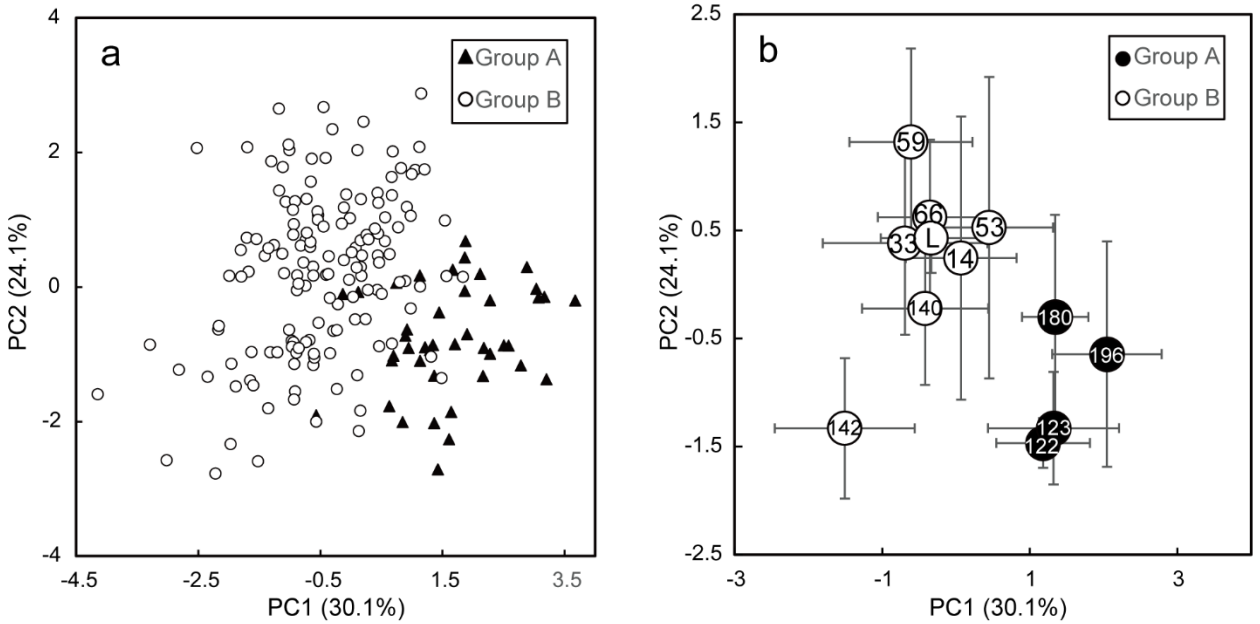


Fig. 2.5. Principal Component Analysis (PCA) based on six morphological characters. a The Principal components of 191 *L. aequinoctialis* complex individuals. b The principal components averaged by strain. Each number in the circle indicates NGY strain's name, and 'L' indicates Landolt's strain: *L. aequinoctialis* 6746. Error bars represent the standard error.

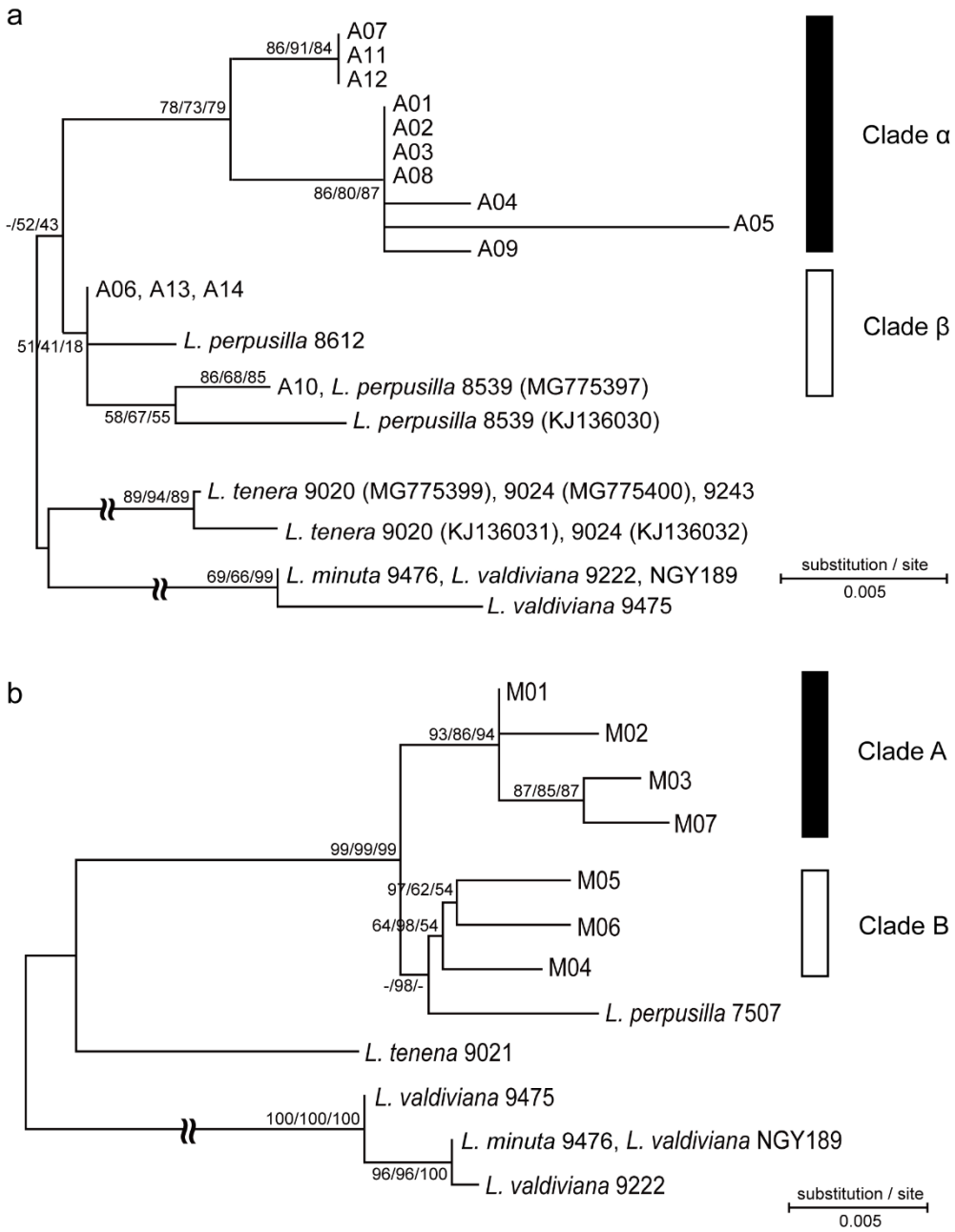


Fig. 2.6. Neighbor joining trees of a *atpF–atpH* and b *matK* based on 421 and 581 aligned characters of cpDNA of 24 and 12 haplotypes of *Lemna aequinoctialis* complex (Table 2.3, 2.4, S2.1) and outgroup. Bootstrap values (ML/MP/NJ) were indicated on branches.

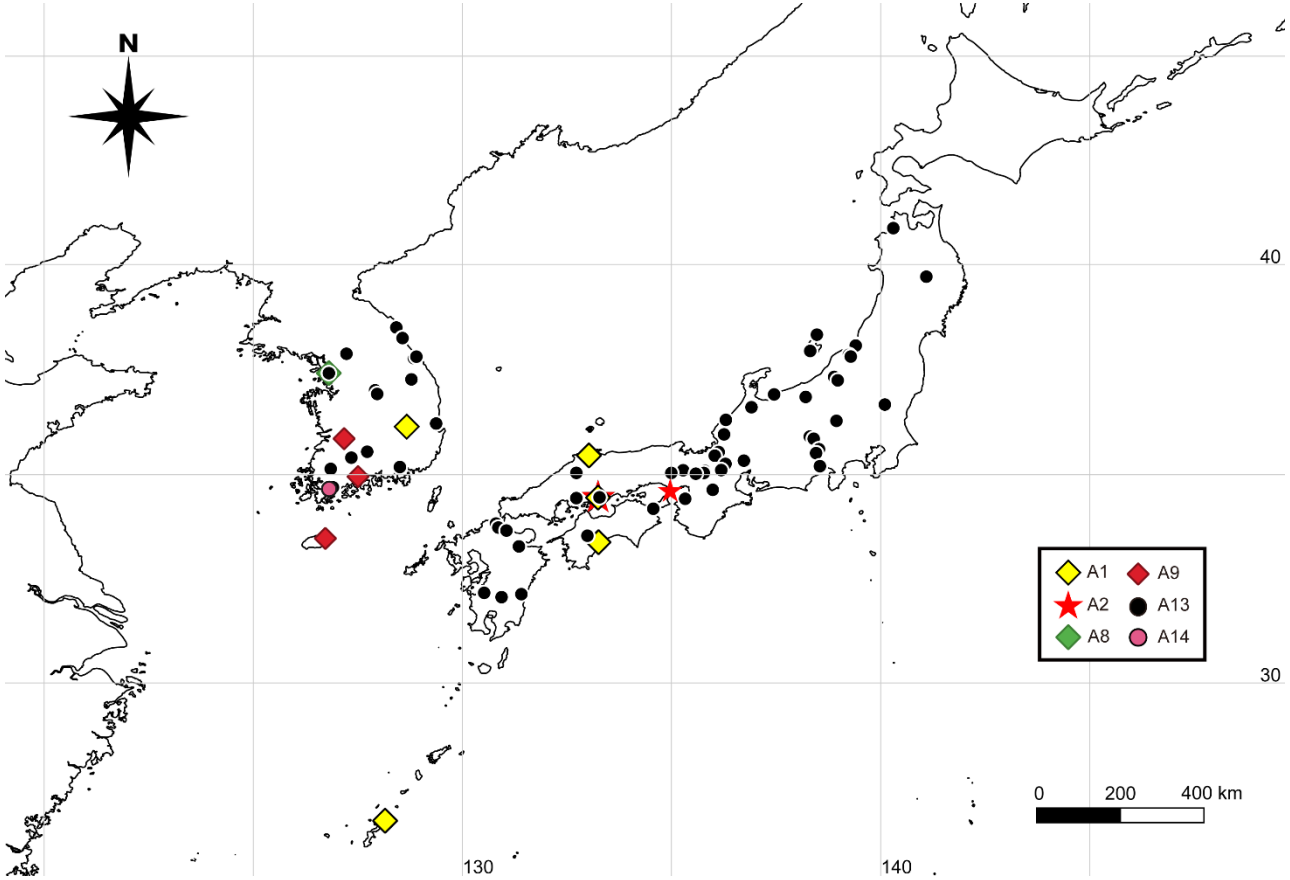


Fig. 2.7. Geographic distribution of the cpDNA eight haplotypes in Japan and Korea. The samples researched only in *atpF-H* were also shown (A1, A2, A8, A9, A13, and A14).

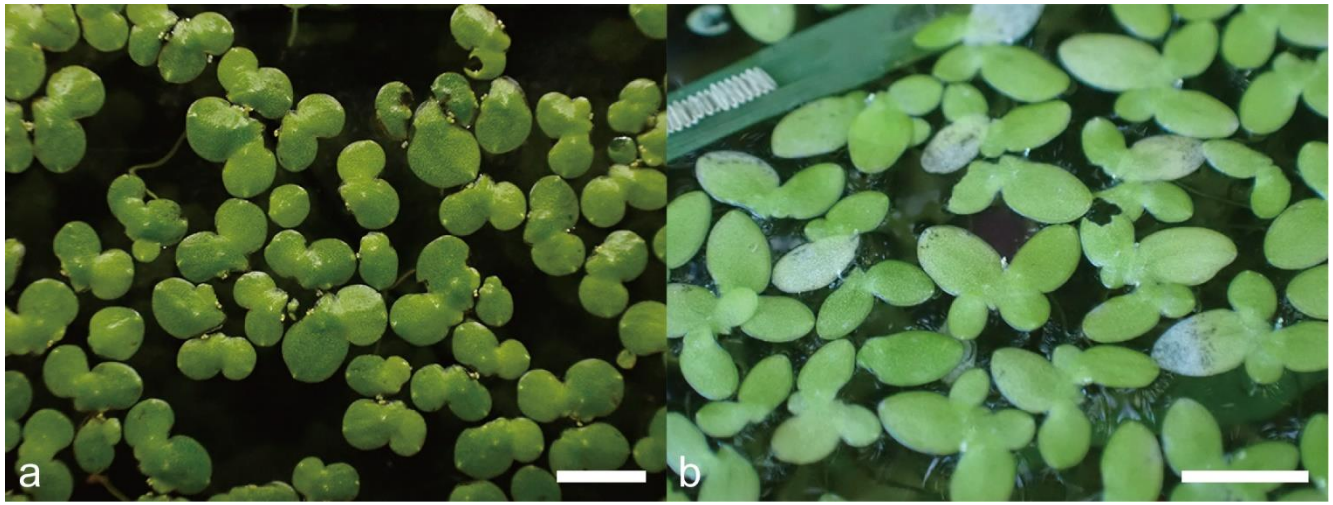


Fig. 2.8. Two groups of *L. aequinoctialis* complex from field trip. The flowering plants were found under natural condition in summer. a. group A. The photograph was taken where NGY196 was collected on September. b. group B. The photograph was taken where NGY156 was collected on August. The scale bar indicates 3mm.

Supplementary Table 2.1. The referred information used in this study. Strain name, locality, haplotype, GenBank accession number were included. Each informations which start character A and M mean sort of haplotype from two chloroplast DNA regions, *atpF-atpH* IGS and *matK*. *Astarisked data (Kim et al. 2017) were not registered in GenBank.

No.	Taxon	Strain	Location of origin	GenBank accession number		Haplotype		Reference
				in *atpF-H*	in *matK*	in *atpF-H*	in *matK*	
1	*L. aequinoctialis*	LC01	China (Lake Chao)	KP017612	KP017666	A3	M1	Tang et al. (2015)
2	*L. aequinoctialis*	LC02	China (Lake Chao)	KP017613	KP017667	A3	M1	Tang et al. (2015)
3	*L. aequinoctialis*	LC05	China (Lake Chao)	KP017616	KP017670	A3	M1	Tang et al. (2015)
4	*L. aequinoctialis*	LC08	China (Lake Chao)	KP017619	KP017673	A3	M1	Tang et al. (2015)
5	*L. aequinoctialis*	LC10	China (Lake Chao)	KP017621	KP017675	A3	M1	Tang et al. (2015)
6	*L. aequinoctialis*	LC12	China (Lake Chao)	KP017623	KP017677	A3	M1	Tang et al. (2015)
7	*L. aequinoctialis*	LC14	China (Lake Chao)	KP017625	KP017679	A4	M1	Tang et al. (2015)
8	*L. aequinoctialis*	LC16	China (Lake Chao)	KP017627	KP017681	A3	M1	Tang et al. (2015)
9	*L. aequinoctialis*	LC18	China (Lake Chao)	KP017629	KP017683	A3	M1	Tang et al. (2015)
10	*L. aequinoctialis*	LC22	China (Lake Chao)	KP017633	KP017686	A3	M1	Tang et al. (2015)
11	*L. aequinoctialis*	LC24	China (Lake Chao)	KP017635	KP017688	A5	M1	Tang et al. (2015)
12	*L. aequinoctialis*	LC25	China (Lake Chao)	KP017636	KP017689	A3	M1	Tang et al. (2015)
13	*L. aequinoctialis*	LC26	China (Lake Chao)	KP017637	KP017690	A3	M1	Tang et al. (2015)
14	*L. aequinoctialis*	LC29	China (Lake Chao)	KP017640	KP017693	A3	M1	Tang et al. (2015)
15	*L. aequinoctialis*	LC31	China (Lake Chao)	KP017642	KP017694	A3	M1	Tang et al. (2015)
16	*L. aequinoctialis*	LC33	China (Lake Chao)	KP017644	KP017695	A3	M1	Tang et al. (2015)
17	*L. aequinoctialis*	LC34	China (Lake Chao)	KP017645	KP017696	A3	M1	Tang et al. (2015)
18	*L. aequinoctialis*	LC35	China (Lake Chao)	KP017646	KP017697	A3	M2	Tang et al. (2015)
19	*L. aequinoctialis*	LC38	China (Lake Chao)	KP017649	KP017700	A3	M1	Tang et al. (2015)
20	*L. aequinoctialis*	LC39	China (Lake Chao)	KP017650	KP017701	A3	M1	Tang et al. (2015)
21	*L. aequinoctialis*	LC41	China (Lake Chao)	KP017652	KP017703	A3	M1	Tang et al. (2015)
22	*L. aequinoctialis*	LC43	China (Lake Chao)	KP017654	KP017705	A3	M1	Tang et al. (2015)
23	*L. aequinoctialis*	LC44	China (Lake Chao)	KP017655	KP017706	A3	M1	Tang et al. (2015)
24	*L. aequinoctialis*	LC45	China (Lake Chao)	KP017656	KP017707	A3	M1	Tang et al. (2015)
25	*L. aequinoctialis*	LC47	China (Lake Chao)	KP017658	KP017708	A3	M1	Tang et al. (2015)
26	*L. aequinoctialis*	LC50	China (Lake Chao)	KP017661	KP017710	A3	M1	Tang et al. (2015)
27	*L. aequinoctialis*	LC51	China (Lake Chao)	KP017662	KP017711	A3	M1	Tang et al. (2015)
28	*L. aequinoctialis*	LC52	China (Lake Chao)	KP017663	KP017712	A3	M1	Tang et al. (2015)
29	*L. aequinoctialis*	LC53	China (Lake Chao)	KP017664	KP017713	A3	M1	Tang et al. (2015)
30	*L. aequinoctialis*	DW1301-3	Chengmai, Hainan, China	KJ630527		A7		Xu et al. (2015)

Supplementary Table 2.1. continued.

31	*L. aequinoctialis*	DW2301-5	Lingshui, Hainan, China	KJ630548		A12		Xu et al. (2015)
32	*L. aequinoctialis*	DW2401-2	Lingshui, Hainan, China	KJ630549		A12		Xu et al. (2015)
33	*L. aequinoctialis*	DW2601-1	Lingshui, Hainan, China	KJ630552		A12		Xu et al. (2015)
34	*L. aequinoctialis*	DW3201-3	Dongfang, Hainan, China	KJ630562		A7		Xu et al. (2015)
35	*L. aequinoctialis*	DW3201-4	Dongfang, Hainan, China	KJ630563		A7		Xu et al. (2015)
36	*L. aequinoctialis*	DW3401-4	Sanya, Hainan, China	KJ630565		A7		Xu et al. (2015)
37	*L. aequinoctialis*	L001*	Gangwon-do, Gangreung-shi, Wichoncheon Stream			A13		Kim et al. (2017)
38	*L. aequinoctialis*	L002*	Gangwon-do, Yeongwol-gun, Near Jucheon High Sch.			A13		Kim et al. (2017)
39	*L. aequinoctialis*	L003*	Gangwon-do, Wonju-shi, Munmakgyo			A13		Kim et al. (2017)
40	*L. aequinoctialis*	L009*	Gyeonggi-do, Pocheon-shi, Gunnae-myeon, rice paddy			A13		Kim et al. (2017)
41	*L. aequinoctialis*	L011*	Gyeonggi-do, Pocheon-shi, Myeongsan-ri			A13		Kim et al. (2017)
42	*L. aequinoctialis*	L012*	Gyeonggi-do, Siheung-shi, Gwan-gok pond			A13		Kim et al. (2017)
43	*L. aequinoctialis*	L013*	Gyeonggi-do, Siheung-shi, Yeonkkot Thema park			A8		Kim et al. (2017)
44	*L. aequinoctialis*	L019*	Chungbuk-do, Chungju-shi, Neung-Am Wetland Park			A13		Kim et al. (2017)
45	*L. aequinoctialis*	L025*	Gyeongbuk-do, Chilgok-gun, Yeong-O-gyo			A1		Kim et al. (2017)
46	*L. aequinoctialis*	L026*	Gyeongbuk-do, Chilgok-gun, Sinnamgyo			A13		Kim et al. (2017)
47	*L. aequinoctialis*	L036*	Jeonbuk-do, Jeonju-shi, Deokjin Park			A9		Kim et al. (2017)
48	*L. aequinoctialis*	L037*	Gwangju Metropolitan City, Jeonpyeongje Pond			A13		Kim et al. (2017)
49	*L. aequinoctialis*	L039*	Jeonnam-do, Gangjin-gun, Tamjin-gang River			A14		Kim et al. (2017)
50	*L. aequinoctialis*	L040*	Jeonnam-do, Jangheung-gun, Jangheung-gyo			A13		Kim et al. (2017)
51	*L. aequinoctialis*	L041*	Jeonnam-do, Suncheon-Shi, Kkum-ui-dari Bridge			A9		Kim et al. (2017)

Supplementary Table 2.1. continued.

52	*L. aequinoctialis*	L042*	Jeonbuk-do, Namwon-shi, Yocheon Stream			A13		Kim et al. (2017)
53	*L. aequinoctialis*	L043*	Gyeongnam-do, Ham-yang-gun, Sangrim Forest			A13		Kim et al. (2017)
54	*L. aequinoctialis*	L044*	Gyeongnam-do, Changwon-shi, Taebong-ri			A13		Kim et al. (2017)
55	*L. aequinoctialis*	L046*	Jeju-do, Jeju-shi, Geum-Oreum Hillside small pond 1			A9		Kim et al. (2017)
56	*L. aequinoctialis*	7339	Bubanza, Bujumbura, Burundi	MG775387		A11		Bog et al. (2020a)
57	*L. aequinoctialis*	9526	India, AP, Hyderabad, Lingampally	MG775388		A12		Bog et al. (2020a)
58	*L. aequinoctialis*	4a	Lake Tai, China (31°25′49.78″ N, 120°17′15.65″ E)	KF726148	KF726226	A3	M1	Tang et al. (2014)
59	*L. aequinoctialis*	7a	Lake Tai, China (31°25′41.33″ N, 120°25′30.75″ E)	KF726151	KF726229	A3	M1	Tang et al. (2014)
60	*L. aequinoctialis*	8a	Lake Tai, China (31°25′05.87″ N, 120°25′03.34″ E)	KF726153	KF726231	A3	M1	Tang et al. (2014)
61	*L. aequinoctialis*	12b	Lake Tai, China (31°24′11.53″ N, 120°24′30.22″ E)	KF726158	KF726236	A3	M1	Tang et al. (2014)
62	*L. aequinoctialis*	13a	Lake Tai, China (31°23′37.25″ N, 120°24′27.58″ E)	KF726159	KF726237	A3	M1	Tang et al. (2014)
63	*L. aequinoctialis*	16b	Lake Tai, China (31°22′23.36″ N, 120°23′11.14″ E)	KF726164	KF726242	A3	M1	Tang et al. (2014)
64	*L. aequinoctialis*	17b	Lake Tai, China (31°21′37.89″ N, 120°21′53.80″ E)	KF726166	KF726244	A3	M1	Tang et al. (2014)
65	*L. aequinoctialis*	18a	Lake Tai, China (31°20′34.27″ N, 120°19′40.07″ E)	KF726167	KF726245	A3	M1	Tang et al. (2014)
66	*L. aequinoctialis*	20a	Lake Tai, China (31°19′36.94″ N, 120°20′35.99″ E)	KF726170	KF726248	A3	M1	Tang et al. (2014)
67	*L. aequinoctialis*	21a	Lake Tai, China (31°18′28.40″ N, 120°21′16.31″ E)	KF726174	KF726252	A3	M1	Tang et al. (2014)
68	*L. aequinoctialis*	24a	Lake Tai, China (31°31′45.78″ N, 120°13′36.17″ E)	KF726178	KF726256	A3	M1	Tang et al. (2014)
69	*L. aequinoctialis*	26a	Lake Tai, China (31°27′57.68″ N, 120°04′15.57″ E)	KF726181	KF726259	A3	M1	Tang et al. (2014)

Supplementary Table 2.1. continued.

70	*L. aequinoctialis*	28a	Lake Tai, China (31°28′15.84″ N, 120°03′51.10″ E)	KF726184	KF726262	A3	M1	Tang et al. (2014)
71	*L. aequinoctialis*	29c	Lake Tai, China (31°25′40.13″ N, 120°00′44.11″ E)	KF726188	KF726266	A3	M1	Tang et al. (2014)
72	*L. aequinoctialis*	30a	Lake Tai, China (31°24′59.96″ N, 120°00′42.08″ E)	KF726189	KF726267	A3	M1	Tang et al. (2014)
73	*L. aequinoctialis*	34a	Lake Tai, China (31°20′36.82″ N, 119°56′22.31″ E)	KF726195	KF726273	A3	M1	Tang et al. (2014)
74	*L. aequinoctialis*	41a	Lake Tai, China (31°05′18.70″ N, 119°57′17.95″ E)	KF726203	KF726281	A3	M1	Tang et al. (2014)
75	*L. aequinoctialis*	43a	Lake Tai, China (31°03′17.50″ N, 119°58′55.40″ E)	KF726206	KF726284	A3	M1	Tang et al. (2014)
76	*L. aequinoctialis*	44a	Lake Tai, China (31°01′47.39″ N, 119°59′40.99″ E)	KF726207	KF726285	A3	M1	Tang et al. (2014)
77	*L. aequinoctialis*	50b	Lake Tai, China (30°55′21.34″ N, 120°15′50.98″ E)	KF726214	KF726292	A3	M1	Tang et al. (2014)
78	*L. aequinoctialis*	52b	Lake Tai, China (30°57′49.92″ N, 120°24′05.97″ E)	KF726218	KF726296	A3	M1	Tang et al. (2014)
79	*L. aequinoctialis*	53a	Lake Tai, China (30°58′49.96″ N, 120°26′16.92″ E)	KF726219	KF726297	A3	M1	Tang et al. (2014)
80	*L. aequinoctialis*	55a	Lake Tai, China (31°04′43.74″ N, 120°35′02.21″ E)	KF726222	KF726300	A3	M1	Tang et al. (2014)
81	*L. aequinoctialis*	OSBAR 000207	unknown		MH551728		M5	Neubig et al. (unpublished)
82	*L. aequinoctialis*	OSBAR 000287	unknown		MH551801		M5	Neubig et al. (unpublished)
83	*L. aequinoctialis*	7671	Brazil		AY034190		M3	Les et al. (2002)
84	*L. aequinoctialis*	6612	Fresno Co., Centerville, California, USA	GU454215	GU454146	A3	M1	Wang et al. (2010)
85	*L. aequinoctialis*	6746	Merced Co., Plainsburg, California, USA	GU454216	GU454147	A6	M4	Wang et al. (2010)
86	*L. aequinoctialis*	7126	Travis Co., Austin (univeresity pond), Texas, USA	GU454217	GU454148	A10	M4	Wang et al. (2010)
87	*L. perpusilla*	7507	USA		AY034191			Les et al. (2002)

Supplementary Table 2.1. continued.

88	*L. perpusilla*	8539	Chesapeake, VA, USA	KJ136030				Borisjuk et al. (2015)
89	*L. perpusilla*	8539	Chesapeake, VA, USA	MG775397				Bog et al. (unpublished)
90	*L. perpusilla*	8612	Princetown, Mercer Co., NJ, USA	MG775398				Bog et al. (unpublished)
91	*L. tenera*	9020	Lake Condor, NT, Australia	KJ136031				Borisjuk et al. (2015)
92	*L. tenera*	9020	Lake Condor, NT, Australia	MG775399				Bog et al. (unpublished)
93	*L. tenera*	9021	Australia		AY034189			Les et al. (2002)
94	*L. tenera*	9024	Nancar Billabong, NT, Australia	KJ136032				Borisjuk et al. (2015)
95	*L. tenera*	9024	Nancar Billabong, NT, Australia	MG775400				Bog et al. (unpublished)

Supplementary Table 2.2. *Lemna aequinoctialis* complex strains used in this study. "+" mark indicates that the strain was used in each analysis

	Flower mature timing	Ability of self-fertilization	Morphometry		PCA	DNA analysis
			induced by Benzoic acid	induced by photoperiodic control		
NGY14	+	+	+	+	+	+
NGY33	+	+	+	+	+	+
NGY53				+	+	+
NGY59	+	+	+	+	+	+
NGY66				+	+	+
NGY122	+	+	+		+	+
NGY123	+	+	+		+	+
NGY128	+	+	+			+
NGY140				+	+	+
NGY142	+	+	+		+	+
NGY180	+	+	+		+	+
NGY196	+	+	+		+	+
NGY302	+	+				+
6746	+	+	+		+	+

Supplementary Table 2.3. The rate of two types of physiology in flowering season in 11 strains of *L. aequinoctialis* complex. The flowers were observed every 12 hours for seven days under dissecting microscope.

Group	Strain name	Homogyny frond number	Protogyny frond number	Protogyny frond rate (%)	Homogyny frond rate (%)	Bloomed frond number	All observed frond number
A	NGY122	3	79	96.34	3.66	82	82
A	NGY123	0	21	100	0	21	48
A	NGY128	1	8	88.89	11.11	9	19
A	NGY180	1	61	98.39	1.61	62	65
A	NGY196	8	23	74.19	25.81	31	34
A	NGY302	2	27	93.10	6.90	29	32
B	NGY14	37	2	5.13	94.87	39	39
B	NGY33	25	0	0	100	25	28
B	NGY59	37	1	2.63	97.37	38	42
B	NGY142	31	2	6.06	93.94	33	33
B	6746	73	0	0	100	73	73

Supplementary Table 2.4. Morphological measurements of two groups, all size was meas ured in millimeters. *, Student's t-test; **, Welch's test; ***, Wilcoxon test; n. s., no sig nificant.

a) Frond

Characters	Group A	Group B	Statistical test
Number	3.81 ± 1.78 (n = 108)	4.37 ± 2.04 (n = 166)	$P = 0.0323$***
Length	2.60 ± 0.27 (n = 107)	2.48 ± 0.86 (n = 164)	$P < 0.0001$***
Width	1.77 ± 0.21 (n = 107)	1.60 ± 0.41 (n = 149)	$P < 0.0001$**
Thickness	0.63 ± 0.09 (n = 103)	0.57 ± 0.12 (n = 162)	$P < 0.0001$***
Vein number	2.50 ± 1.01 (n = 107)	2.35 ± 1.23 (n = 164)	n. s.***
Papillae number	4.17 ± 1.01 (n = 107)	4.00 ± 2.14 (n = 166)	$P = 0.0009$***

b) Root

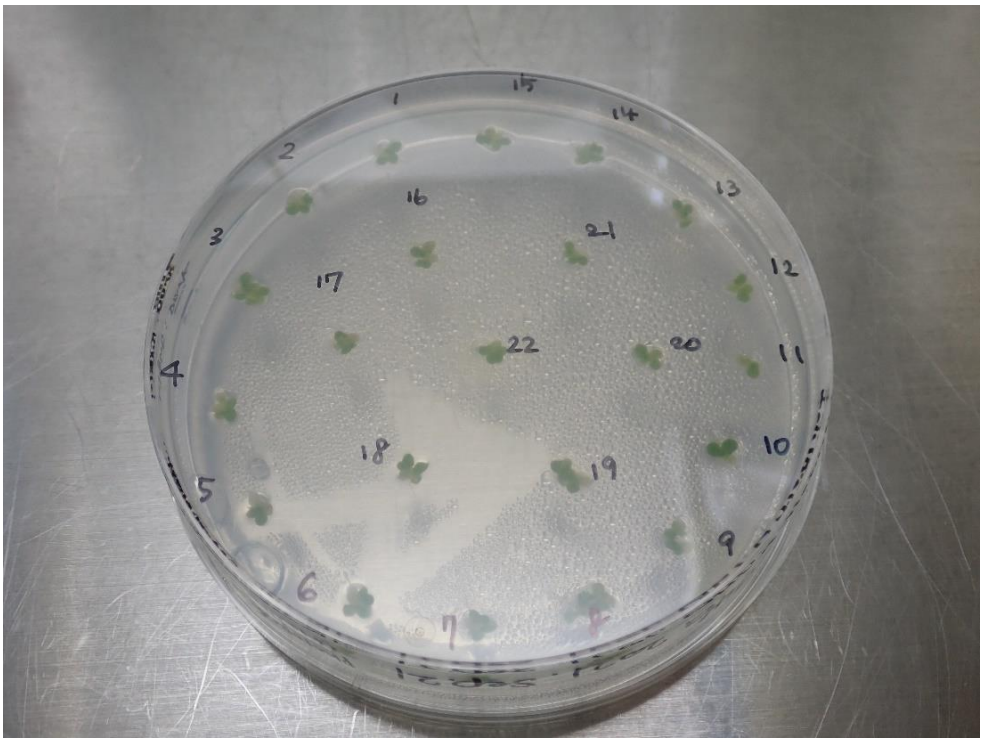
Characters	Group A	Group B	Statistical test
Length	12.48 ± 6.96 (n = 107)	9.34 ± 5.80 (n = 161)	$P = 0.0001$***
Diameter	0.14 ± 0.02 (n = 107)	0.15 ± 0.03 (n = 161)	$P < 0.0001$***
Cap length	1.92 ± 0.38 (n = 94)	1.76 ± 0.32 (n = 66)	$P = 0.0056$*
Cap diameter	0.19 ± 0.02 (n = 94)	0.17 ± 0.03 (n = 66)	$P = 0.0002$***
Sheath length	0.40 ± 0.11 (n = 106)	0.33 ± 0.09 (n = 68)	$P < 0.0001$*
Sheath width	0.37 ± 0.09 (n = 106)	0.35 ± 0.09 (n = 67)	n. s.***

c) Flower

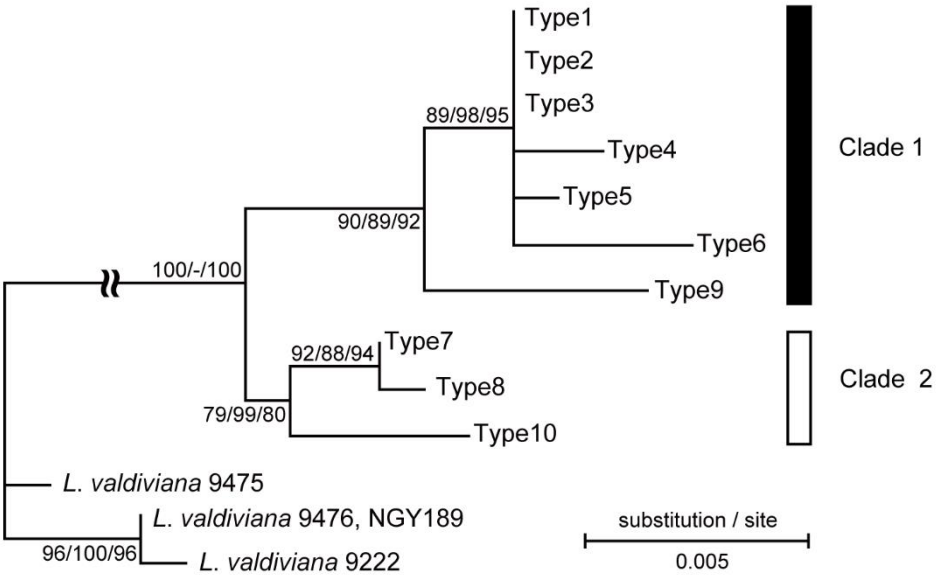
Characters	Group A	Group B	Statistical test
Spathe length	0.54 ± 0.08 (n = 101)	0.51 ± 0.10 (n = 162)	$P = 0.0028$*
Spathe diameter	0.71 ± 0.12 (n = 101)	0.63 ± 0.13 (n = 162)	$P < 0.0001$*
Stigma width	0.13 ± 0.03 (n = 73)	0.14 ± 0.03 (n = 155)	$P = 0.0121$***
Style length	0.24 ± 0.07 (n = 71)	0.20 ± 0.06 (n = 154)	$P < 0.0001$***
Style diameter	0.11 ± 0.02 (n = 101)	0.11 ± 0.02 (n = 162)	$P = 0.006$***
Ovary length	0.22 ± 0.05 (n = 105)	0.26 ± 0.05 (n = 162)	$P < 0.0001$***
Ovary diameter	0.24 ± 0.05 (n = 105)	0.24 ± 0.04 (n = 163)	n. s.***
Filament length	0.60 ± 0.13 (n = 101)	0.52 ± 0.14 (n = 156)	$P < 0.0001$*
Filament diameter	0.14 ± 0.03 (n = 102)	0.12 ± 0.02 (n = 161)	$P = 0.0001$***
Anther length	0.18 ± 0.03 (n = 104)	0.14 ± 0.02 (n = 162)	$P < 0.0001$***
Anther diameter	0.15 ± 0.02 (n = 104)	0.12 ± 0.02 (n = 162)	$P < 0.0001$***

Supplementary Table 2.5. The first three principal components of six morphological characters from 12 strains of *L. aequinoctialis* c omplex. Eigenvalues and percentages of variance and cumulative variance to the total variance for the first three principal compone nts are also listed.

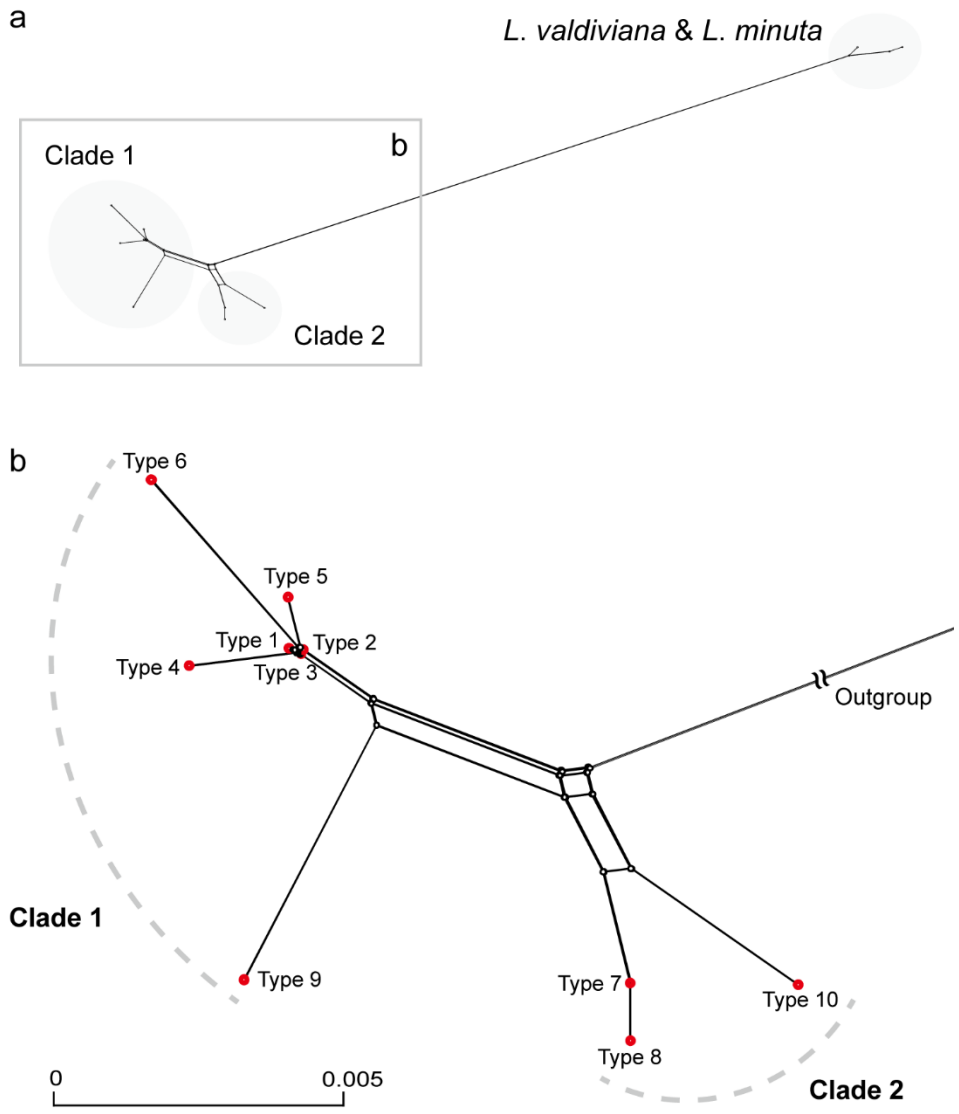
Character	PCA 1	PCA 2	PCA 3
spathe length	0.58113	0.25529	-0.61037
spathe diameter	0.72030	0.12393	-0.13832
ovary length	-0.15063	0.80933	-0.20813
stigma width	0.17787	0.71178	0.38858
filament diameter	0.60592	0.12173	0.57109
anther length	0.72620	-0.43526	0.01079
Eigenvalue	1.8054	1.4464	0.9123
Variance %	30.09	24.11	15.20
Cumulative % of variance	30.09	54.20	69.40



Supplementary Fig. 2.1. Landolt's *L. aequinoctialis* no. 6746. 22 fronds were fixed on semisolid 20^{-1} strength NH4+ free Hutner's medium including 0.3% agar, 1% sucrose, and 10 μM Benzoic acid. We numbered each frond under the 90 × 20 mm sized petri dish.



Supplementary Fig. 2.2. Neighbor joining tree based on 1071 aligned characters of cpDNA (concatenated regions of *atpF-H* and *matK*) of 13 haplotypes of *Lemna aequinoctialis* complex (Table 3, 4) and outgroup. Bootstrap values (ML /MP / NJ) were indicated on branches.



Supplementary Fig. 2.3. Neighbor-net splits tree based on 1071 aligned characters of cpDNA (concatenated regions of *atpF-H* and *matK*) of 13 haplotypes of *Lemna aequinoctialis* complex (Table 3, 4) and outgroup.

Table 3.1. Sampling localities and population codes of *Lemna trisulca* with voucher data.

Population code	Sampling locality	Latitude & Longitude	Voucher	Strain
IT	Iturup Island	45.0645, 147.84	SAP T. Fukuda 2012-542	
KC	Esashi city, Hokkaido Pref.	45.14097, 142.2806		NGY L.tri2
KN	Akkeshi city, Hokkaido Pref.	43.16053, 145.24848		NGY206
TC	Nikko-shi, Tochigi Pref.	36.79641, 139.4281		NGYL.tri3, NGY203
SO	Sunto city, Shizuoka Pref.	35.105, 138.9012		NGY L.tri1
KS	Kitasaku city, Nagano Pref.	36.37083, 138.5867		NGY170
NG	Nagano-shi, Nagano Pref.	36.71467, 138.1018		NGY172

Table 3.2. Genetic diversity of seven populations of *Lemna trisulca* in Japan based on 381 SNP loci.

Population code	N	*Na*	*Ne*	No. PA	*He*	*Ho*	*uHe*	F_{IS} = (*He*-*Ho*)/*He*
IT	1	0.709	0.709	0.016	0.043	0.087	0.087	-1.000
KC	4	1.213	1.174	0.031	0.112	0.195	0.141	-0.743
KN	1	0.780	0.780	0.037	0.056	0.113	0.113	-1.000
TC	2	1.102	1.079	0.068	0.106	0.186	0.170	-0.764
SO	5	1.236	1.181	0.092	0.127	0.223	0.155	-0.751
KS	9	1.336	1.210	0.073	0.120	0.180	0.134	-0.496
NG	9	1.423	1.247	0.121	0.144	0.188	0.160	-0.311

N, number of sampled plant; *Na*, number of different alleles per locus; *Ne*, number of effective alleles per locus; PA, Private Alleles; *He*, expected heterozygosity; *Ho*, observed heterozygosity; *uHe*, unbiased expected heterozygosity; F_{IS}, inbreeding coefficient

Table 3.3. Morphological characters observed in the present study and the result. N means observed plant number.

	Population code	N	Root presence	vein number
1	IT	1	present	1 or 3
2	KC	5	present	1 or 3
3	KN	3	present	3
4	TC	11	absent	1 or 3
5	SO	3	absent	1 or 3
6	KS	10	absent	1
7	NG	10	absent	1 or 3

Supplementary Table 3.1. Genetic distance pairwise matrix between 35 individuals of *L. trisulca*.

	Pop. Code	1	2	3	4	5	6	7	8	9	10	11	12
1	IT-1	0.000											
2	KC-1	0.126	0.000										
3	KC-2	0.135	0.097	0.000									
4	KC-3	0.141	0.095	0.098	0.000								
5	KC-4	0.133	0.118	0.117	0.107	0.000							
6	KN-1	0.193	0.158	0.175	0.160	0.185	0.000						
7	TC-1	0.144	0.152	0.155	0.159	0.169	0.184	0.000					
8	TC-2	0.166	0.179	0.193	0.183	0.210	0.194	0.149	0.000				
9	SO-1	0.186	0.183	0.180	0.184	0.205	0.226	0.215	0.246	0.000			
10	SO-2	0.161	0.166	0.167	0.171	0.179	0.221	0.197	0.227	0.124	0.000		
11	SO-3	0.156	0.161	0.163	0.163	0.183	0.213	0.195	0.247	0.133	0.133	0.000	
12	SO-4	0.165	0.173	0.170	0.183	0.191	0.235	0.172	0.219	0.125	0.123	0.112	0.000
13	SO-5	0.166	0.167	0.176	0.190	0.195	0.219	0.206	0.223	0.139	0.124	0.133	0.136
14	KS-1	0.320	0.306	0.313	0.302	0.311	0.345	0.334	0.340	0.342	0.348	0.363	0.360
15	KS-2	0.317	0.299	0.303	0.296	0.310	0.326	0.348	0.340	0.341	0.345	0.356	0.361
16	KS-3	0.285	0.290	0.289	0.277	0.297	0.337	0.330	0.331	0.323	0.344	0.343	0.336
17	KS-4	0.328	0.317	0.321	0.318	0.326	0.359	0.353	0.370	0.364	0.377	0.369	0.369
18	KS-5	0.326	0.311	0.304	0.294	0.312	0.347	0.353	0.370	0.355	0.352	0.353	0.358
19	KS-6	0.326	0.310	0.322	0.303	0.326	0.347	0.352	0.380	0.358	0.360	0.363	0.376
20	KS-7	0.315	0.314	0.319	0.313	0.323	0.335	0.358	0.373	0.360	0.376	0.365	0.362
21	KS-8	0.314	0.303	0.304	0.293	0.314	0.339	0.329	0.355	0.354	0.350	0.357	0.380
22	KS-9	0.323	0.302	0.305	0.294	0.305	0.348	0.350	0.369	0.343	0.357	0.351	0.369
23	KS-9	0.337	0.335	0.341	0.333	0.340	0.371	0.361	0.376	0.364	0.372	0.376	0.385
24	KS-9	0.318	0.305	0.320	0.304	0.320	0.324	0.333	0.353	0.346	0.350	0.357	0.354
25	NG-1	0.292	0.288	0.282	0.281	0.281	0.306	0.323	0.323	0.333	0.333	0.330	0.327
26	NG-2	0.292	0.291	0.288	0.275	0.295	0.314	0.318	0.318	0.328	0.333	0.332	0.333
27	NG-3	0.297	0.295	0.301	0.296	0.300	0.292	0.321	0.318	0.339	0.322	0.322	0.332
28	NG-4	0.302	0.298	0.306	0.294	0.300	0.321	0.333	0.335	0.332	0.339	0.329	0.346
29	NG-5	0.311	0.310	0.310	0.295	0.298	0.328	0.346	0.333	0.325	0.345	0.317	0.331
30	NG-6	0.291	0.294	0.296	0.274	0.270	0.306	0.322	0.314	0.329	0.326	0.314	0.312
31	NG-7	0.289	0.291	0.280	0.277	0.293	0.316	0.331	0.335	0.309	0.323	0.319	0.333
32	NG-8	0.279	0.281	0.289	0.276	0.276	0.330	0.322	0.317	0.330	0.319	0.340	0.320
33	NG-9	0.298	0.295	0.298	0.286	0.298	0.328	0.322	0.333	0.330	0.347	0.329	0.333
34	NG-9	0.301	0.323	0.318	0.307	0.318	0.340	0.351	0.331	0.337	0.347	0.345	0.353
35	NG-9	0.291	0.282	0.279	0.271	0.285	0.317	0.319	0.314	0.317	0.312	0.303	0.322

Supplementary Table 3.1. continued

	Pop. Code	13	14	15	16	17	18	19	20	21	22	23	24
1	IT-1												
2	KC-1												
3	KC-2												
4	KC-3												
5	KC-4												
6	KN-1												
7	TC-1												
8	TC-2												
9	SO-1												
10	SO-2												
11	SO-3												
12	SO-4												
13	SO-5	0.000											
14	KS-1	0.359	0.000										
15	KS-2	0.354	0.090	0.000									
16	KS-3	0.344	0.091	0.097	0.000								
17	KS-4	0.369	0.103	0.096	0.104	0.000							
18	KS-5	0.351	0.098	0.093	0.106	0.101	0.000						
19	KS-6	0.360	0.091	0.092	0.101	0.107	0.100	0.000					
20	KS-7	0.366	0.103	0.099	0.113	0.113	0.104	0.107	0.000				
21	KS-8	0.370	0.093	0.083	0.095	0.099	0.087	0.087	0.105	0.000			
22	KS-9	0.358	0.098	0.087	0.104	0.107	0.093	0.094	0.110	0.083	0.000		
23	KS-9	0.381	0.105	0.110	0.102	0.108	0.105	0.112	0.124	0.098	0.113	0.000	
24	KS-9	0.359	0.106	0.094	0.097	0.113	0.105	0.100	0.109	0.096	0.100	0.103	0.000
25	NG-1	0.333	0.142	0.147	0.142	0.184	0.133	0.150	0.145	0.144	0.156	0.164	0.122
26	NG-2	0.336	0.140	0.136	0.150	0.163	0.139	0.147	0.143	0.146	0.152	0.149	0.143
27	NG-3	0.332	0.148	0.162	0.154	0.175	0.155	0.151	0.158	0.154	0.173	0.168	0.160
28	NG-4	0.333	0.168	0.164	0.176	0.181	0.163	0.164	0.170	0.165	0.174	0.188	0.145
29	NG-5	0.321	0.162	0.165	0.162	0.186	0.147	0.163	0.167	0.160	0.175	0.173	0.154
30	NG-6	0.326	0.157	0.166	0.154	0.168	0.154	0.158	0.152	0.152	0.170	0.160	0.147
31	NG-7	0.324	0.152	0.157	0.154	0.173	0.143	0.163	0.160	0.148	0.165	0.163	0.153
32	NG-8	0.333	0.152	0.158	0.162	0.164	0.147	0.154	0.144	0.152	0.172	0.171	0.140
33	NG-9	0.331	0.146	0.152	0.144	0.158	0.136	0.158	0.155	0.144	0.157	0.165	0.146
34	NG-9	0.347	0.144	0.153	0.149	0.172	0.149	0.164	0.165	0.146	0.154	0.171	0.150
35	NG-9	0.312	0.144	0.144	0.157	0.158	0.141	0.148	0.153	0.146	0.161	0.167	0.143

Supplementary Table 3.1. continued

	Pop. Code	25	26	27	28	29	30	31	32	33	34	35
1	IT-1											
2	KC-1											
3	KC-2											
4	KC-3											
5	KC-4											
6	KN-1											
7	TC-1											
8	TC-2											
9	SO-1											
10	SO-2											
11	SO-3											
12	SO-4											
13	SO-5											
14	KS-1											
15	KS-2											
16	KS-3											
17	KS-4											
18	KS-5											
19	KS-6											
20	KS-7											
21	KS-8											
22	KS-9											
23	KS-9											
24	KS-9											
25	NG-1	0.000										
26	NG-2	0.095	0.000									
27	NG-3	0.088	0.105	0.000								
28	NG-4	0.111	0.120	0.111	0.000							
29	NG-5	0.112	0.115	0.124	0.124	0.000						
30	NG-6	0.097	0.117	0.112	0.124	0.120	0.000					
31	NG-7	0.093	0.111	0.102	0.113	0.099	0.118	0.000				
32	NG-8	0.099	0.100	0.118	0.103	0.127	0.105	0.096	0.000			
33	NG-9	0.106	0.112	0.106	0.106	0.106	0.119	0.107	0.094	0.000		
34	NG-9	0.108	0.112	0.129	0.129	0.140	0.106	0.110	0.107	0.109	0.000	
35	NG-9	0.084	0.103	0.097	0.099	0.103	0.106	0.091	0.087	0.089	0.102	0.000

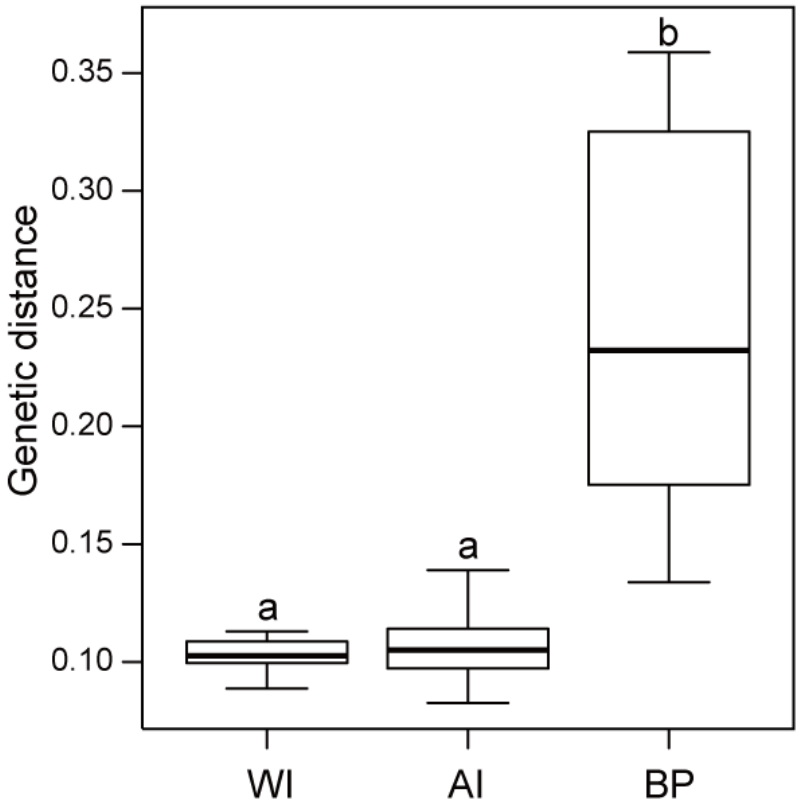


Figure 3.1. Boxplot of genetic distance pair under three conditions; WI, within individual; AI, among individuals in same population; BP, between populations. Same letters above the boxplot indicate that they are not significantly different ($p > 0.05$) by Student's test and Welch's test. For each box plot, top bar indicates maximum value, top end of the box indicates third quartile, the middle bar indicates median value, and the circles indicate possible outliers.

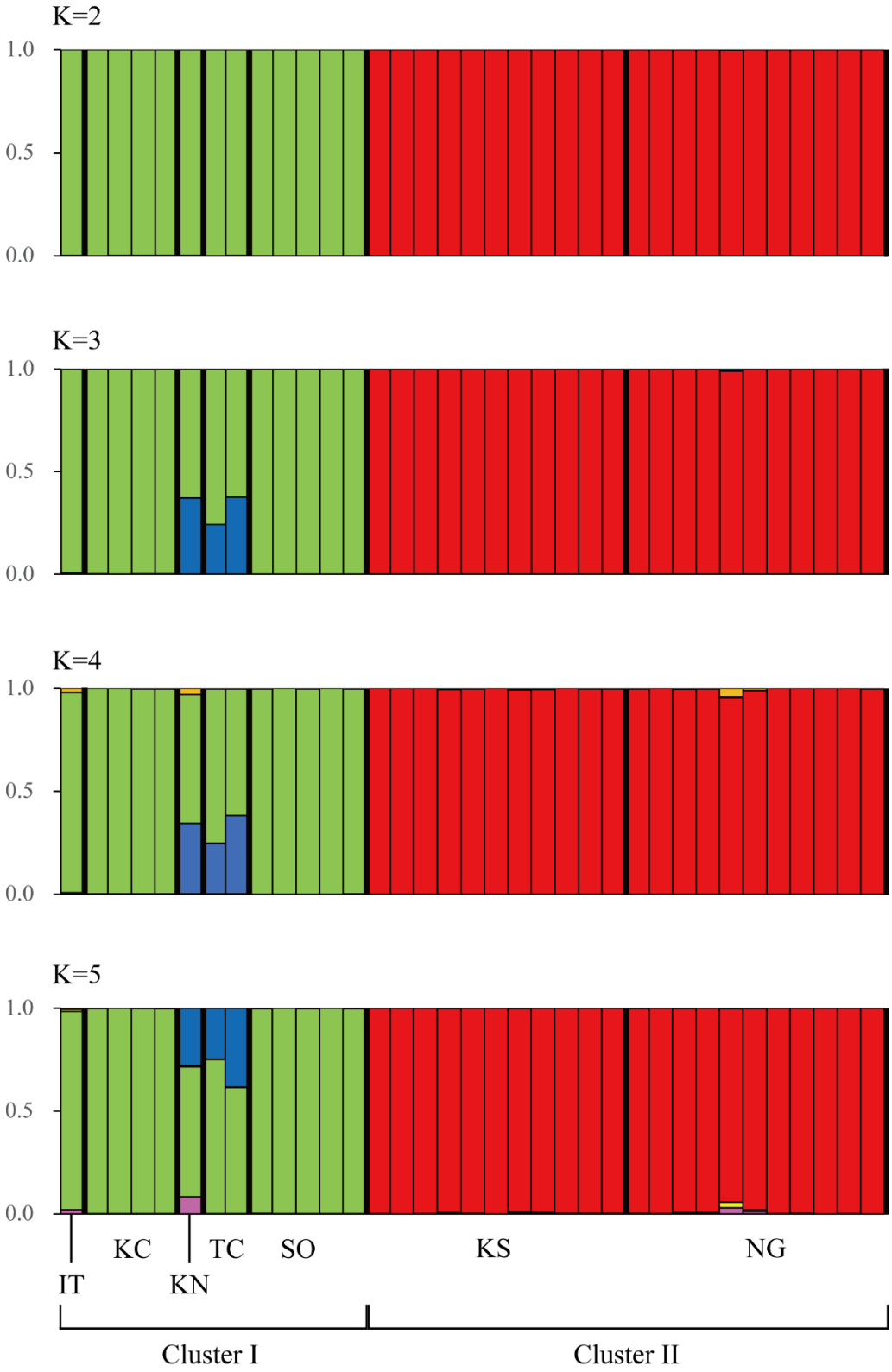


Figure 3.2. Structure analysis of K=2–5 based on 381 SNP loci of Japanese *L. trisulca*. Population codes were written under each bar plot.

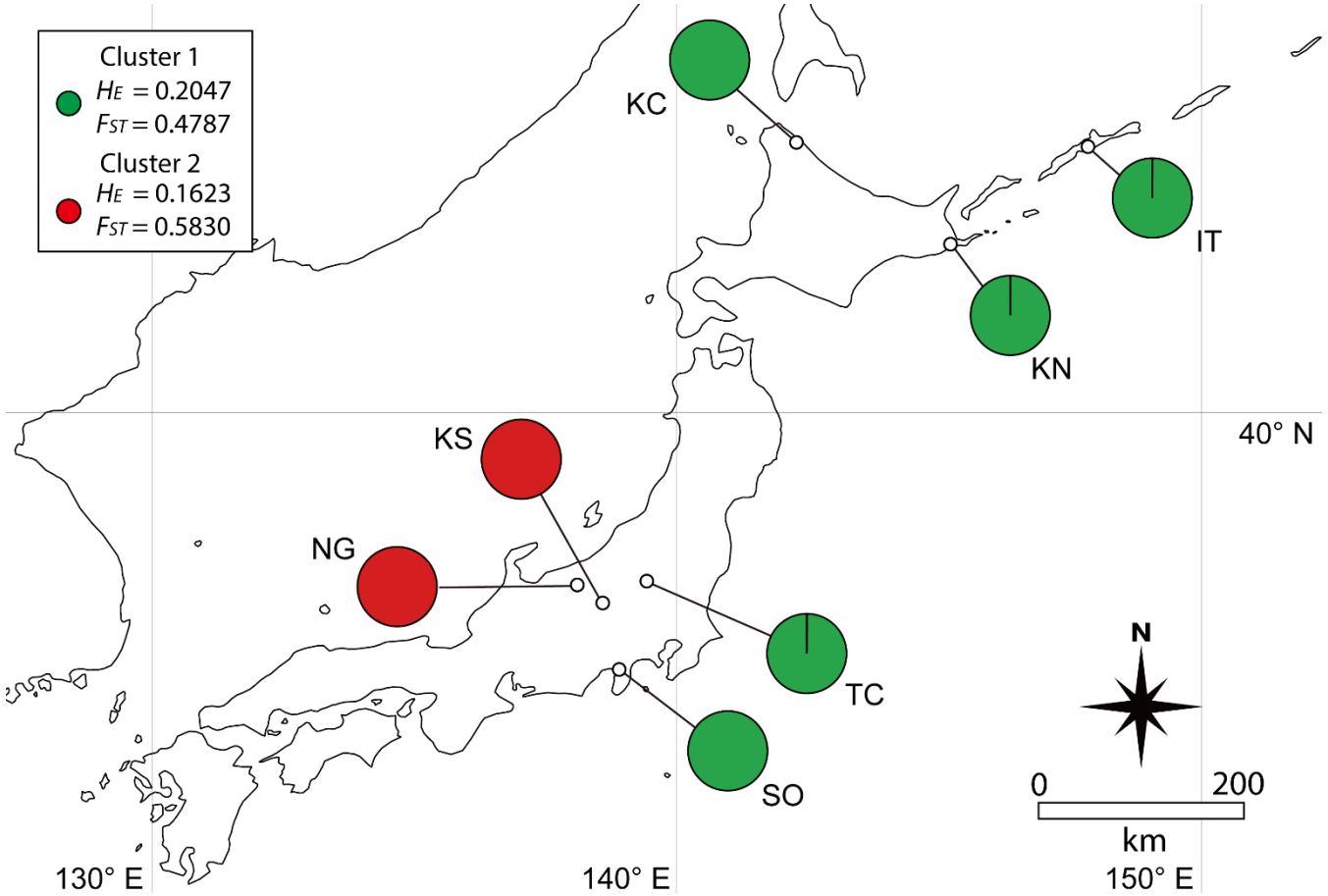


Figure 3.3. Geographical gnetic structure of *L. trisulca* under the ancestry (k) is 2 based on 381 SNP loci. Pie charts indicate attribution ratio to each cluster in populations. H_E and F_{ST} values of each cluster were also shown.

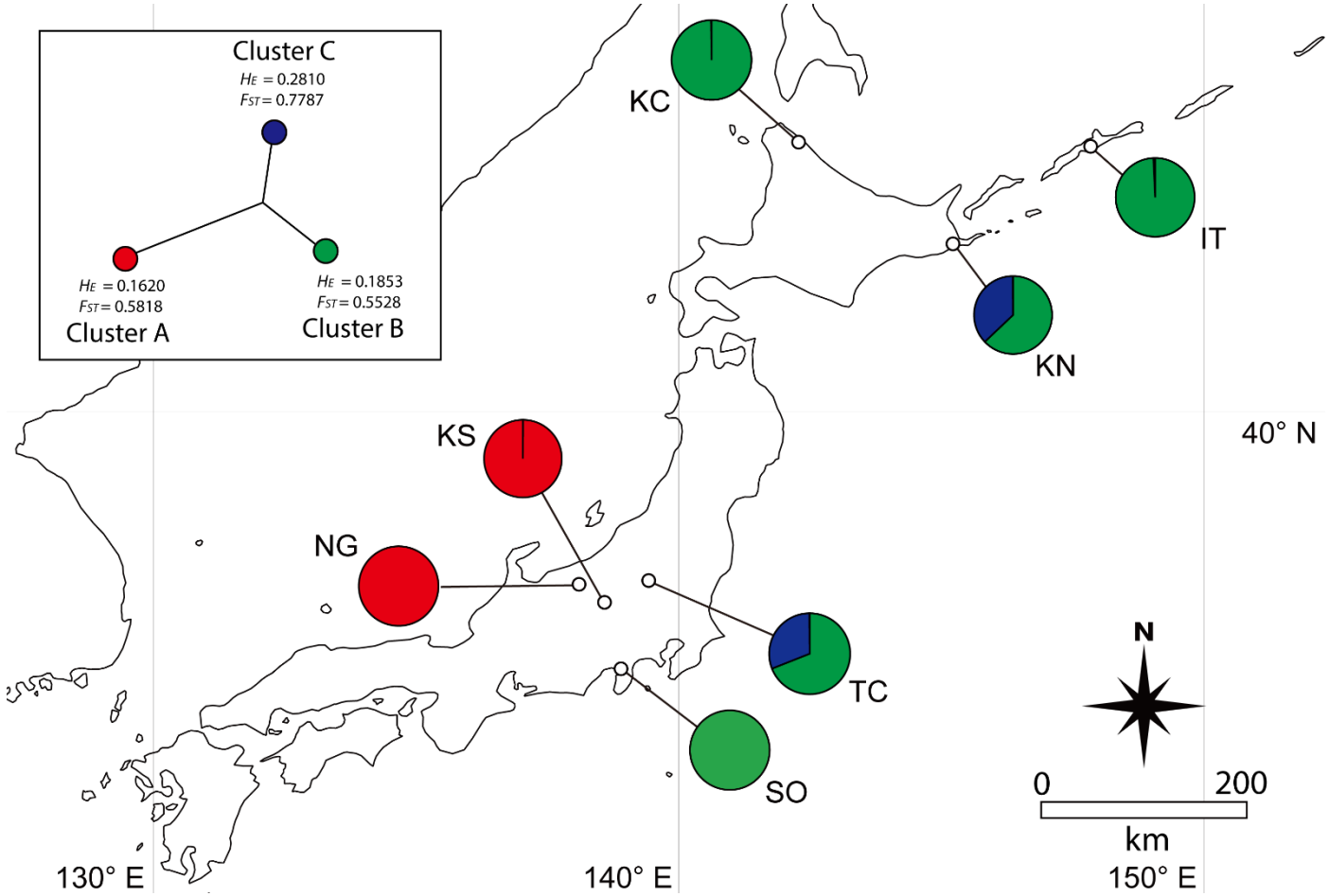


Figure 3.4. Geographical gnetic structure of *L. trisulca* under the ancestry (k) is 3 based on 381 SNP loci. Pie charts indicate attribution ratio to each cluster in populations. H_E and F_{ST} values of each cluster were also shown.

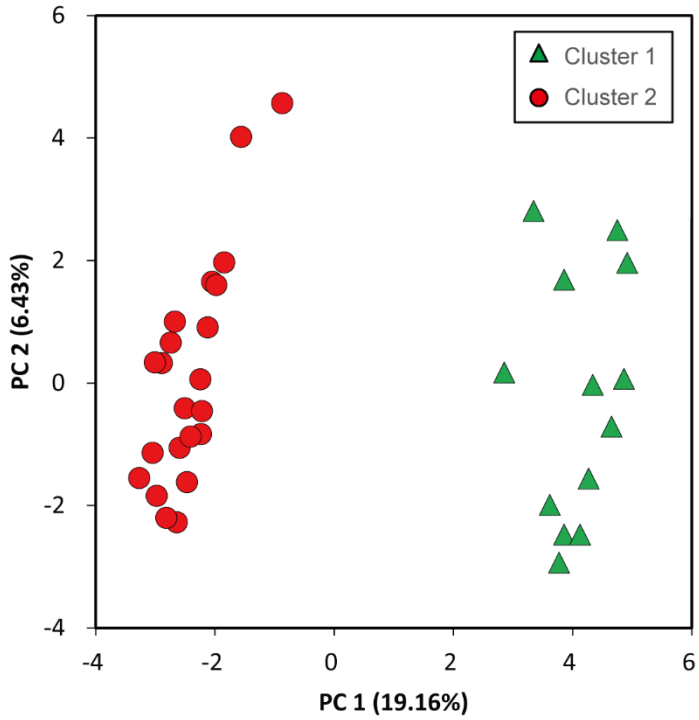


Figure 3.5. Results of principal coordinate analysis (PCoA) based on 381 SNP loci among seven populations of *L. trisulca*. Symbols indicate individuals of cluster 1 and 2 (k=2). Proportion of total variance of each axis was also shown.

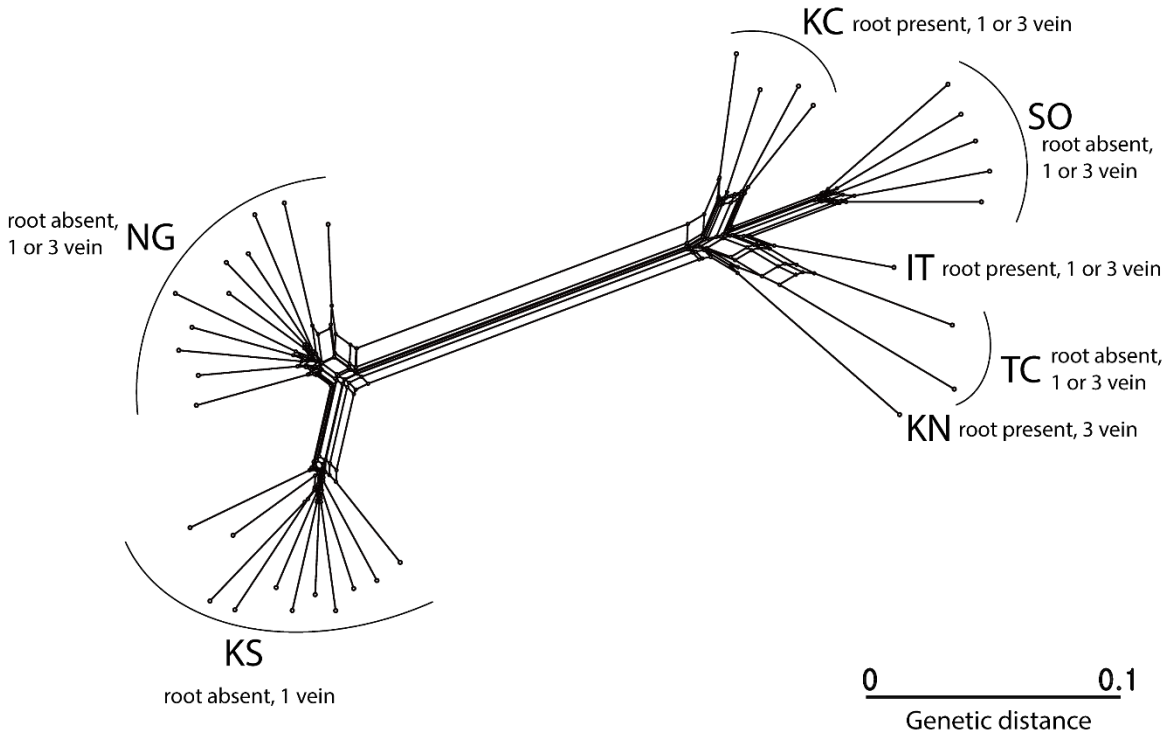
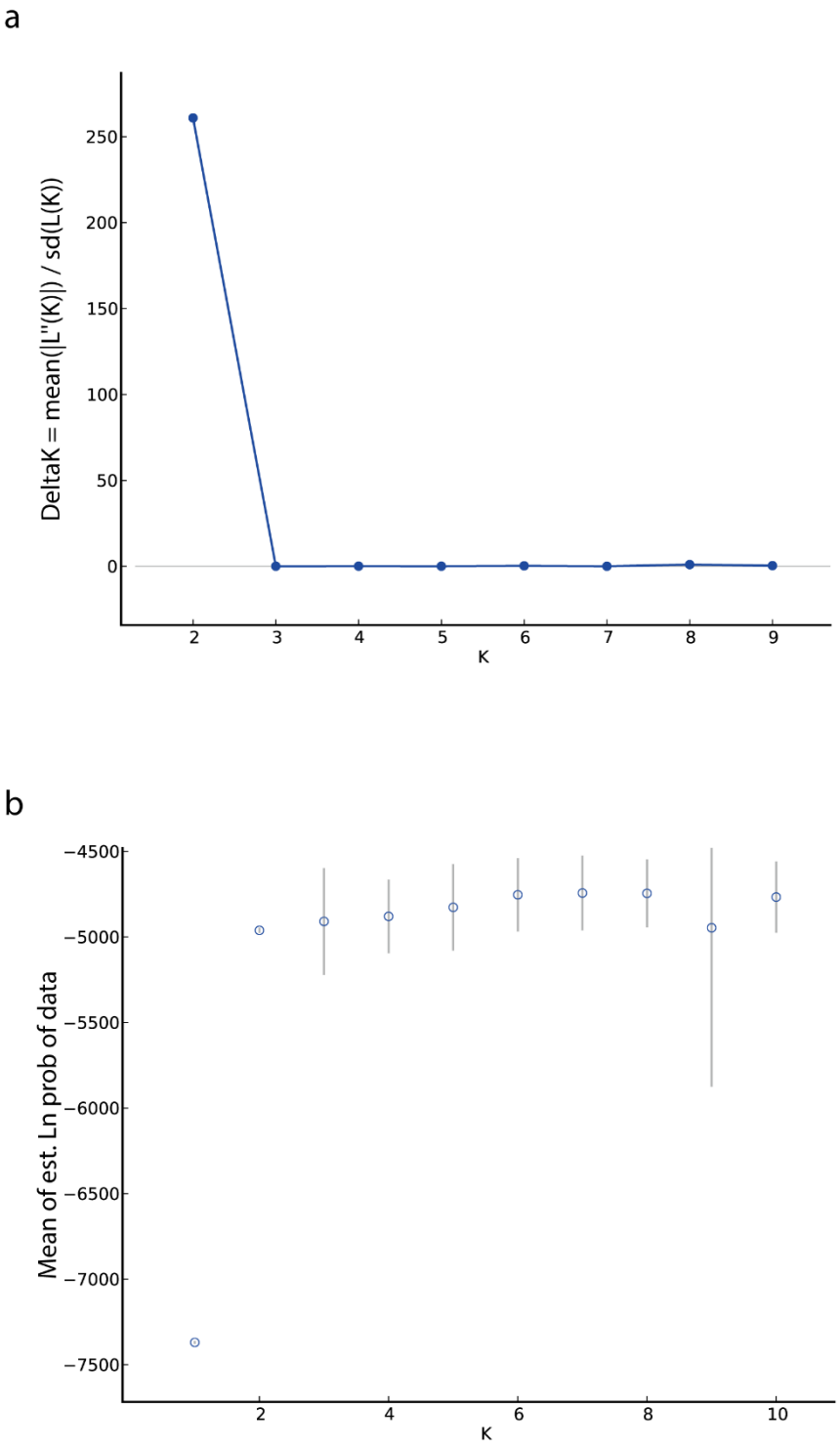


Figure 3.6. Neighbor-net Network based on 381 SNP loci among 35 samples from seven populations of *L. trisulca*. The scale bar indicates genetic distance.



Supplemantary Figure 3.1. Results of STRUCTURE analysis. (a) Delta *K* values and (b) L(K) (mean± SD) of STRUCTURE analysis.

Table 4.1. Collected *Lemna* strains and their information. The population code, origin of the plants, haplotypes were provided. NGY is Lemnoideae species living strain code in Niigata University. KHB (The sample order is same as in STRUCTURE analysis.)

Sample No.	Population code	Country	Sampling locality	N	E	Collection Date	Strain / Voucher	Haplotype
1	P1	Japan	Hokkaido Pref.: Sunagawa-shi	43.50413	141.9075	07. Aug. 2020	NGY152	M
2							NGY152-st3	
3							NGY153-st1	
4							NGY153-st2	
5	P2	Japan	Aomori Pref. Hirosaki-shi	37.93278	138.3132	26. Aug. 2020	NGY158-11	M
6	P3	Japan	Niigata Pref.: Itoigawa-shi	36.91476	137.9006	07. Oct. 2019	NGY114	M
7							NGY114(1)-c2	
8	P4	Japan	Nagano Pref.: Nagano-shi	36.71467	138.1018	11. Sep. 2020	NGY173	M
9							NGY173-st3	
10	P5	Japan	Nagano Pref.: Kitasaku District	36.37083	138.5867	11. Sep. 2020	NGY171	M
11							NGY171-st3	
12	P6	Japan	Yamanashi Pref.: Minamitsuru District	35.46083	138.8331	11. Sep. 2020	NGY175	M
13	P7	Japan	Hokkaido Pref.: Esashi District	45.12947	142.3307	11. Aug. 2019	NGY86	M
14	P8	Japan	Hokkaido Pref.: Kawakami District	38.41144	140.3524	30. Jul. 2019	NGY82	M
15	P9	Japan	Hokkaido Pref.: Soya District	37.93781	139.361	15. Aug. 2019	NGY88	M
16	P2	Japan	Aomori Pref. Hirosaki-shi	37.93278	138.3132	26. Aug. 2020	NGY155	M
17							NGY155-12	
18							NGY158-12	
19	P10	Japan	Yamagata Pref.: Higashine-shi	38.41144	140.3524	25 Aug. 2019	NGY100	M
20	P11	Japan	Niigata Pref.: Shibata-shi	37.93781	139.361	28. May 2019	NGY7	M
21	P12	Japan	Niigata Pref.: Sado-shi	37.93278	138.3132	28. Jun. 2019	NGY26	M

Table 4.1. continued

Sample No.	Population code	Country	Sampling locality	N	E	Collection Date	Strain / Voucher	Haplotype
22							NGY27	
23	P13	Japan	Niigata Pref.: Sado-shi	37.9316	138.3122	29. Jun. 2019	NGY29	M
24	P14	Japan	Niigata Pref.: Sado-shi	37.93139	138.3111	29. Jun. 2019	NGY30	M
25	P15	Japan	Niigata Pref.: Sado-shi	37.93132	138.3103	29. Jun. 2019	NGY28	M
26	P16	Japan	Niigata Pref.: Mitsuke-shi	37.53378	138.9825	17. Oct. 2019	NGY116	M
27	P17	Japan	Niigata Pref.: Nagaoka-shi	37.40598	139.0173	13. Jun. 2020	NGY127	M
28	P18	Japan	Niigata Pref.: Uonuma-shi	37.37563	139.0359	19. Oct. 2019	NGY117	M
29	P19	Japan	Niigata Pref.: Ojiya-shi	37.31711	138.778	20. Jun. 2019	NGY22	M
30	P20	Japan	Niigata Pref.: Joetsu-shi	37.24178	138.3611	19. Sep. 2020	NGY201	M
31	P21	Japan	Niigata Pref.: Nagaoka-shi	37.23864	138.8511	10. Oct. 2019	NGY115	M
32	P22	Japan	Niigata Pref.: Minamiuonuma District	37.07447	138.8767	11. Aug. 2019	NGY84	M
33	P23	Japan	Niigata Pref.: Joetsu-shi	36.97947	138.2291	05. Oct. 2019	NGY111	M
34	P24	Japan	Tokyo	35.7	139.7	21. Jun. 2019	NGY23	M
35	P25	Korea	Gangwon-do: Samcheok-si	37.46603	129.1674	13. Aug. 2011	KHB1346517	J
36	P26	Japan	Niigata Pref.: Joetsu-shi	37.11	138.12	24. Jul. 2019	NGY69-2	J
37	P27	Japan	Niigata Pref.: Joetsu-shi	37	138	24. Jul. 2019	NGY69	J
38	P28	Japan	Niigata Pref.: Joetsu-shi	37	138	05. Oct. 2019	NGY109	J
39							NGY109(4)	
40							NGY109(1)	
41							NGY109(2)	
42							NGY109(3)	

Table 4.1. continued

Sample No.	Population code	Country	Sampling locality	N	E	Collection Date	Strain / Voucher	Haplotype
43							NGY109(1)-c1	
44							NGY109(1)-c2	
45							NGY109(1)-c3	
46	P29	Japan	Tochigi Pref.: Nikko-shi	36.80796	139.4243	29. Sep. 2020.	NGY204	J
47							NGY204-st2	
48	P30	Japan	Gifu Pref.: Hida-shi	36.24331	137.1813	29. Jul. 2020.	NGY149	J
49							NGY149-st2	
50							NGY149-st3	
51	P31	Japan	Fukui Pref.: Fukui-shi	36.0601	136.2567	20. Jul. 2019	NGY78	J
52	P32	Korea	Chungcheongnam-do: Gongju-si	36	127	25. Jun. 2015	KHB1145701	J
53	P33	Japan	Yamanashi Pref.: Chuo-shi	35.60279	138.5159	16. Jul. 2019	NGY55	J
54	P34	Japan	Tottori Pref.: Tohaku District	35.4675	133.9333	22. Sep. 2020	NGY179	J
55							NGY179-2	
56							NGY191-1	
57							NGY191-2	
58	P35	Japan	Shimane Pref.: Matsue-shi	35.435	133.0219	22. Sep. 2020	NGY194	J
59							NGY194-2	
60							NGY194-3	
61							NGY180-11	
62							NGY180-12	
63							NGY180-13	

Table 4.1. continued

Sample No.	Population code	Country	Sampling locality	N	E	Collection Date	Strain / Voucher	Haplotype
64	P36	Japan	Gyeongsangnam-do: Sancheong-gun	35.30059	127.9409	04. Nov. 2012	KHB1420047	J
65	P37	Japan	Kanagawa Pref.: Yokosuka-shi	35.23793	139.6064	10. Jun. 2010	S4	J
66	P38	Japan	Hyogo Pref.: Awaji-shi	34.52554	134.9848	25. Sep. 2020	NGY188	J
67							NGY188-2	
68							NGY198	
69	P39	Japan	Hiroshima Pref.: Onomichi-shi	34.42806	133.24	24. Sep. 2020	NGY195	J
70							NGY184-12	
71	P40	Japan	Hyogo Pref.: Sumoto-shi	34.40694	134.8367	25. Sep. 2020	NGY187	J
72							NGY187-11	
73							NGY187-12	
74	P41	Japan	Niigata Pref.: Mitsuke-shi	34	138	Nov.14 2019	NGY119	J
75	P42	Japan	Kochi Pref.: Kochi-shi	33.5425	133.4939	06. Jul. 2019	NGY38	J
76	P43	Japan	Kochi Pref.: Kochi-shi	33.54247	133.4935	06. Jul. 2019	NGY37	J
77	P44	Japan	Kochi Pref.: Tosa-shi	33.5105	133.4199	31 Jul. 2012	NGY207	J
78	P45	Japan	Oita Pref.: Yufu-shi	33.22606	131.3495	25 Jul. 2019	NGY68	J
79	P46	Korea	Jeju-do: Seogwipo-si	33	126	05. Sep. 2013	KHB1435284	J
80	P47	Japan	Hokkaido Pref.: Sunagawa-shi	43.50413	141.9075	07. Aug. 2020	NGY153	T
81	P48	Japan	Hokkaido Pref.: Akkeshi District	43.06242	145.0863	07. Aug. 2020	NGY151	T
82							NGY151-st2	
83							NGY151-st3	
84	P49	Japan	Miyagi Pref.: Higashimatsushima-shi	38.34676	141.1539	28. Aug. 2019	NGY96	T

Table 4.1. continued

Sample No.	Population code	Country	Sampling locality	N	E	Collection Date	Strain / Voucher	Haplotype
85	P50	Japan	Miyagi Pref.: Higashimatsushima-shi	38.3308	141.1384	17. Sep. 2020	NGY200-1	T
86							NGY200-2	
87							NGY200-3	
88	P51	Japan	Nagano Pref.: Suwa-shi	36.05361	138.1122	30. Nov. 2019	NGY120	M
81	P48	Japan	Hokkaido Pref.: Akkeshi District	43.06242	145.0863	07. Aug. 2020	NGY151	T
82							NGY151-st2	
83							NGY151-st3	
84	P49	Japan	Miyagi Pref.: Higashimatsushima-shi	38.34676	141.1539	28. Aug. 2019	NGY96	T
85	P50	Japan	Miyagi Pref.: Higashimatsushima-shi	38.3308	141.1384	17. Sep. 2020	NGY200-1	T
86							NGY200-2	
87							NGY200-3	
88	P51	Japan	Nagano Pref.: Suwa-shi	36.05361	138.1122	30. Nov. 2019	NGY120	M

Table 4.2. Japanese section *Lemna* strains used in the present study. The cluster which sample belongs to and the samples' haplotype were provided. "+" mark indicates that the strain was used in each analysis. "–" mark indicates that the strain was not used in the present study. The used plant number (n) was added in parenthesis.

Strain	Cluster / Haplotype	Flower development observation	Pollen number	Pollen viability	Morphometric analysis	PCA
NGY114-1	subcluster a / M	+ (n = 17)	+ (n = 12)	+ (n = 8)	+ (n = 6)	+ (n = 6)
NGY152-11	subcluster a / M	+ (n = 12)	+ (n = 5)	+ (n = 5)	–	–
NGY153-14	subcluster a / M	+ (n = 38)	–	–	+ (n = 6)	+ (n = 6)
NGY173-11	subcluster a / M	+ (n = 42)	+ (n = 11)	+ (n = 8)	+ (n = 6)	+ (n = 6)
NGY86-1	subcluster b / M	+ (n = 12)	–	–	–	–
NGY117-2	subcluster b / M	+ (n = 10)	–	–	–	
NGY187-12	cluster B / J	+ (n = 23)	+ (n = 10)	+ (n = 10)	+ (n = 6)	+ (n = 6)
NGY109-2	cluster B / J	+ (n = 6)	–	–	+ (n = 6)	+ (n = 6)
NGY151	cluster C / T	+ (n = 19)	+ (n = 10)	+ (n = 10)	+ (n = 5)	+ (n = 5)
NGY200-13	cluster C / T	–	+ (n = 6)	+ (n = 6)	+ (n = 10)	+ (n = 10)
L. japonica 7182	– / J	+ (n = 34)	+ (n = 10)	+ (n = 10)	+ (n = 10)	+ (n = 10)

Table 4.3. Variation sites of three haplotypes of *atpF-H* intergenic spacer (IGS) chloroplast DNA of Japan native section *Lemna*. The numbers indicate variable in the region.

	SNP position of *matK*															
									1	1	1	1	2	2	2	3
	3	6	6	6	6	6	8	8	0	2	6	9	1	2	4	8
Haplotype	0	4	5	6	7	8	6	7	2	9	2	1	3	8	9	8
M	C	-	T	T	T	T	A	A	A	A	C	G	G	A	-	G
J	C	T	T	T	T	T	A	A	A	A	C	G	G	A	-	G
T	T	-	-	-	-	-	T	T	T	-	T	A	A	G	A	C

Table 4.4. Genetic diversity of Japanese native section *Lemna* collected in this study.

Population code	N	Na	Ne	I	Ho	He	uHe	F
P1	4	*0.463*	*0.458*	*0.097*	*0.131*	*0.069*	*0.084*	*-0.871*
P2	4	*0.461*	*0.448*	*0.096*	*0.127*	*0.068*	*0.082*	*-0.832*
P3	2	*0.443*	*0.437*	*0.098*	*0.134*	*0.070*	*0.102*	*-0.895*
P4	2	*0.441*	*0.436*	*0.095*	*0.134*	*0.068*	*0.103*	*-0.952*
P5	2	*0.896*	*0.894*	*0.010*	*0.010*	*0.007*	*0.011*	*-0.444*
P6	1	*0.792*	*0.792*	*0.007*	*0.010*	*0.005*	*0.010*	*-1.000*
P7	1	*0.772*	*0.772*	*0.009*	*0.013*	*0.006*	*0.013*	*-1.000*
P8	1	*0.770*	*0.770*	*0.005*	*0.008*	*0.004*	*0.008*	*-1.000*
P9	1	*0.724*	*0.724*	*0.007*	*0.010*	*0.005*	*0.010*	*-1.000*
P10	1	*0.628*	*0.628*	*0.004*	*0.005*	*0.003*	*0.005*	*-1.000*
P11	1	*0.496*	*0.496*	*0.002*	*0.003*	*0.001*	*0.003*	*-1.000*
P12	2	*0.901*	*0.897*	*0.014*	*0.018*	*0.010*	*0.016*	*-0.704*
P13	1	*0.691*	*0.691*	*0.011*	*0.015*	*0.008*	*0.015*	*-1.000*
P14	1	*0.790*	*0.790*	*0.007*	*0.010*	*0.005*	*0.010*	*-1.000*
P15	1	*0.787*	*0.787*	*0.005*	*0.008*	*0.004*	*0.008*	*-1.000*
P16	1	*0.671*	*0.671*	*0.011*	*0.015*	*0.008*	*0.015*	*-1.000*
P17	1	*0.841*	*0.841*	*0.014*	*0.020*	*0.010*	*0.020*	*-1.000*
P18	1	*0.843*	*0.843*	*0.009*	*0.013*	*0.006*	*0.013*	*-1.000*
P19	1	*0.772*	*0.772*	*0.014*	*0.020*	*0.010*	*0.020*	*-1.000*
P20	1	*0.818*	*0.818*	*0.009*	*0.013*	*0.006*	*0.013*	*-1.000*
P21	1	*0.777*	*0.777*	*0.009*	*0.013*	*0.006*	*0.013*	*-1.000*
P22	1	*0.448*	*0.448*	*0.004*	*0.005*	*0.003*	*0.005*	*-1.000*
P23	1	*0.590*	*0.590*	*0.012*	*0.018*	*0.009*	*0.018*	*-1.000*
P24	1	*0.770*	*0.770*	*0.007*	*0.010*	*0.005*	*0.010*	*-1.000*
P25	1	*0.347*	*0.347*	*0.012*	*0.018*	*0.009*	*0.018*	*-1.000*
P26	1	*0.922*	*0.922*	*0.063*	*0.091*	*0.046*	*0.091*	*-1.000*
P27	1	*0.962*	*0.962*	*0.067*	*0.096*	*0.048*	*0.096*	*-1.000*
P28	8	*1.157*	*1.131*	*0.100*	*0.126*	*0.070*	*0.076*	*-0.711*
P29	2	*1.058*	*1.046*	*0.077*	*0.101*	*0.054*	*0.088*	*-0.826*
P30	3	*1.094*	*1.086*	*0.085*	*0.118*	*0.061*	*0.080*	*-0.915*
P31	1	*0.957*	*0.957*	*0.067*	*0.096*	*0.048*	*0.096*	*-1.000*
P32	1	*0.446*	*0.446*	*0.021*	*0.030*	*0.015*	*0.030*	*-1.000*
P33	1	*0.985*	*0.985*	*0.072*	*0.104*	*0.052*	*0.104*	*-1.000*
P34	4	*1.137*	*1.124*	*0.096*	*0.123*	*0.068*	*0.085*	*-0.780*
P35	6	*1.154*	*1.128*	*0.099*	*0.121*	*0.069*	*0.080*	*-0.681*
P36	1	*0.620*	*0.620*	*0.032*	*0.046*	*0.023*	*0.046*	*-1.000*

Table 4.4. continued.

Population code	N	Na	Ne	I	Ho	He	uHe	F
P37	1	0.749	0.749	0.047	0.068	0.034	0.068	-1.000
P38	3	1.129	1.124	0.100	0.141	0.072	0.097	-0.947
P39	2	1.094	1.082	0.087	0.116	0.062	0.091	-0.846
P40	3	1.132	1.122	0.093	0.125	0.066	0.085	-0.856
P41	1	0.965	0.965	0.077	0.111	0.056	0.111	-1.000
P42	1	0.868	0.868	0.060	0.086	0.043	0.086	-1.000
P43	1	0.939	0.939	0.063	0.091	0.046	0.091	-1.000
P44	1	0.787	0.787	0.040	0.058	0.029	0.058	-1.000
P45	1	0.929	0.929	0.063	0.091	0.046	0.091	-1.000
P46	1	0.759	0.759	0.051	0.073	0.037	0.073	-1.000
P47	1	0.597	0.597	0.028	0.041	0.020	0.041	-1.000
P48	3	0.699	0.693	0.047	0.063	0.034	0.043	-0.852
P49	1	0.380	0.380	0.005	0.008	0.004	0.008	-1.000
P50	3	0.646	0.645	0.019	0.027	0.014	0.019	-0.939
P51	1	0.803	0.803	0.007	0.010	0.005	0.010	-1.000
Mean		0.782	0.779	0.042	0.058	0.030	0.048	-0.905

Table 4.5. Morphological measurements of three (sub)clades. All size was measured in millimeters w ith two decimal digits. *, Welch's t-test; **, ANOVA or Wilcoxon test; ***; n. s., no significant.

a) Frond

Characters	subcluster a	cluster B	cluster C	Statistical test
Number	5.67 ± 2.52 (n = 18)	4.09 ± 1.15 (n = 22)	2.40 ± 0.63 (n = 15)	$P = 0.0001$***
Length	3.38 ± 0.57 (n = 18)	3.41 ± 0.25 (n = 22)	2.43 ± 0.32 (n = 15)	$P < 0.0001$**
Width	2.31 ± 0.49 (n = 18)	2.25 ± 0.18 (n = 22)	1.69 ± 0.28 (n = 15)	$P < 0.0001$**
Thickness	0.64 ± 0.12 (n = 18)	0.90 ± 0.11 (n = 22)	0.63 ± 0.18 (n = 15)	$P < 0.0001$**
Vein number	2.56 ± 1.58 (n = 18)	0.14 ± 0.64 (n = 22)	0.60 ± 1.24 (n = 15)	$P < 0.0001$**
Papillae number	4.06 ± 2.26 (n = 18)	6.27 ± 1.52 (n = 22)	5.00 ± 0.76 (n = 15)	$P = 0.0011$***
Length/Width	1.48 ± 0.15 (n = 18)	1.52 ± 0.07 (n = 22)	1.45 ± 0.08 (n = 15)	n. s.***

b) Root

Characters	subcluster a	cluster B	cluster C	Statistical test
Length	6.47 ± 3.71 (n = 18)	18 ± 5.12 (n = 22)	6.21 ± 3.46 (n = 15)	$P < 0.0001$**
Diameter	0.12 ± 0.03 (n = 18)	0.17 ± 0.01 (n = 22)	0.14 ± 0.02 (n = 15)	$P < 0.0001$**
Cap length	1.04 ± 0.29 (n = 18)	1.77 ± 0.24 (n = 22)	1.30 ± 0.38 (n = 15)	$P < 0.0001$**
Cap diameter	0.18 ± 0.02 (n = 18)	0.23 ± 0.03 (n = 22)	0.19 ± 0.03 (n = 15)	$P = 0.0001$***

c) Flower

Characters	subcluster a	cluster B	cluster C	Statistical test
Spathe front length	0.19 ± 0.06 (n = 14)	0.23 ± 0.09 (n = 22)	0.28 ± 0.10 (n = 15)	$P = 0.0253$*
Spathe length (total)	0.77 ± 0.09 (n = 18)	0.89 ± 0.19 (n = 22)	0.92 ± 0.18 (n = 15)	$P < 0.0001$**
Spathe diameter	0.80 ± 0.08 (n = 18)	0.75 ± 0.13 (n = 22)	0.81 ± 0.08 (n = 15)	n. s.**
Stigma width	0.19 ± 0.04 (n = 18)	0.23 ± 0.03 (n = 22)	0.15 ± 0.03 (n = 15)	$P < 0.0001$**
Style length	0.37 ± 0.12 (n = 18)	0.40 ± 0.08 (n = 22)	0.32 ± 0.08 (n = 15)	$P = 0.0509$**
Style diameter	0.15 ± 0.02 (n = 18)	0.16 ± 0.02 (n = 22)	0.15 ± 0.03 (n = 15)	$P = 0.0554$***
Ovary length	0.33 ± 0.04 (n = 18)	0.35 ± 0.04 (n = 22)	0.32 ± 0.05 (n = 15)	n. s.**
Ovary diameter	0.34 ± 0.04 (n = 18)	0.34 ± 0.03 (n = 22)	0.29 ± 0.03 (n = 15)	$P = 0.0004$**
Filament length	0.80 ± 0.24 (n = 18)	0.25 ± 0.05 (n = 22)	0.75 ± 0.10 (n = 15)	$P < 0.0001$**
Filament diameter	0.18 ± 0.05 (n = 18)	0.13 ± 0.03 (n = 22)	0.18 ± 0.02 (n = 15)	$P < 0.0001$**
Anther length	0.26 ± 0.02 (n = 18)	0.24 ± 0.01 (n = 22)	0.24 ± 0.03 (n = 15)	$P = 0.0005$**
Anther diameter	0.19 ± 0.03 (n = 18)	0.20 ± 0.02 (n = 22)	0.19 ± 0.03 (n = 15)	n. s.**

Table 4.6. The first three principal components of six morphologic al characters from eight strains of *L. minor*, *L. japonica*, *L. turionifera*. Eigenvalues and percentages of variance and cumulativ e variance to the total variance for the first three principal compo nents are also listed.

Character	PCA 1	PCA 2	PCA 3
Frond number	0.07818	0.70291	-0.07206
Frond thickness	0.49856	-0.23536	0.04521
Ovary diameter	0.32887	0.53755	0.24275
Stigma width	0.48799	0.20747	-0.16804
Stamen length	-0.44999	0.21197	0.6843
Root cap length	0.44334	-0.27126	0.66132
Eigenvalue	2.6987	1.5292	0.5538
Variance %	44.978	25.487	9.23
Cumulative % of variance	44.98	70.47	79.70

Table 4.7. Collected *Lemna* strains (cluster B) and their information. The population code, origin of the plants, haplotypes were provided. NGY is Lemnoideae species living strain code in Niigata University. KHB (The sample order is same as in STRUCTURE analysis.)

Sample No.	Population code	Country	Sampling locality	N	E	Collection Date	Strain / Voucher
1	PJ1	Japan	Niigata Pref.: Joetsu-shi	37	138	20190724	NGY69
2		Japan	Niigata Pref.: Joetsu-shi	37	138	20190724	NGY69-2
3	PJ2	Japan	Niigata Pref.: Joetsu-shi	37	138	20191005	NGY109
4		Japan	Niigata Pref.: Joetsu-shi	37	138	20191005	NGY109(4)
5		Japan	Niigata Pref.: Joetsu-shi	37	138	20191005	NGY109(1)
6		Japan	Niigata Pref.: Joetsu-shi	37	138	20191005	NGY109(2)
7		Japan	Niigata Pref.: Joetsu-shi	37	138	20191005	NGY109(3)
8		Japan	Niigata Pref.: Joetsu-shi	37	138	20191005	NGY109(1)-c1
9		Japan	Niigata Pref.: Joetsu-shi	37	138	20191005	NGY109(1)-c2
10		Japan	Niigata Pref.: Joetsu-shi	37	138	20191005	NGY109(1)-c3
11	PJ3	Japan	Tochigi Pref.: Nikko-shi	36.80796	139.4243	20200929	NGY204-st1
12		Japan	Tochigi Pref.: Nikko-shi	36.80796	139.4243	20200929	NGY204-st2
13	PJ4	Japan	Gifu Pref.: Hida-shi	36.24331	137.1813	20200729	NGY149-st1
14		Japan	Gifu Pref.: Hida-shi	36.24331	137.1813	20200729	NGY149-st2
15		Japan	Gifu Pref.: Hida-shi	36.24331	137.1813	20200729	NGY149-st3
16	PJ5	Japan	Fukui Pref.L: Fukui-shi	36.0601	136.2567	20190720	NGY78
17	PJ6	Japan	Yamanashi Pref.: Chuo-shi	35.60279	138.5159	20190716	NGY55
18	PJ7	Japan	Tottori Pref.: Touhaku-kun	35.4675	133.9333	20200922	NGY179-1
19		Japan	Tottori Pref.: Touhaku-kun	35.4675	133.9333	20200922	NGY179-2
20		Japan	Tottori Pref.: Touhaku-kun	35.4675	133.9333	20200922	NGY191-1
21		Japan	Tottori Pref.: Touhaku-kun	35.4675	133.9333	20200922	NGY191-2
22	PJ8	Japan	Shimane Pref.: Matsue-shi	35.435	133.0219	20200922	NGY194-1
23		Japan	Shimane Pref.: Matsue-shi	35.435	133.0219	20200923	NGY194-2
24		Japan	Shimane Pref.: Matsue-shi	35.435	133.0219	20200924	NGY194-3
25	PJ9	Korea	Gyeongsangnam-do, Sancheong-gun	35.30059	127.9409	20121104	20190614_4
26	PJ10	Japan	Kanagawa Pref.: Yokosuka-shi	35.23793	139.6064	20100610	S4

Table 4.7. continued

Sample No.	Population code	Country	Sampling locality	N	E	Collection Date	Strain / Voucher
27	PJ11	Japan	Hyogo Pref.: Awaji-shi	34.52554	134.9848	20200925	NGY188-1
28		Japan	Hyogo Pref.: Awaji-shi	34.52554	134.9848	20200925	NGY188-2
29		Japan	Hyogo Pref.: Awaji-shi	34.52554	134.9848	20200925	NGY198
30	PJ12	Japan	Hiroshima Pref.: Onomichi-shi	34.42806	133.24	20200924	NGY195-2
31	PJ13	Japan	Hyogo Pref.: Sumoto-shi	34.40694	134.8367	20200925	NGY187
32		Japan	Hyogo Pref.: Sumoto-shi	34.40694	134.8367	20200925	NGY187-11
33		Japan	Hyogo Pref.: Sumoto-shi	34.40694	134.8367	20200925	NGY187-12
34	PJ14	Japan	Shizuoka Pref.: Shizuoka-shi	34	138	20191114	NGY119
35	PJ15	Japan	Kochi Pref.: Kochi-shi	33.54247	133.4935	20190706	NGY37
36		Japan	Kochi Pref.: Kochi-shi	33.5425	133.4939	20190706	NGY38
37	PJ16	Japan	Kochi Pref.: Kochi-shi	33.5105	133.4199	20120731	NGY207
38	PJ17	Japan	Oita Pref.: Yufu-shi	33.22606	131.3495	20190725	NGY68
39	PJ18	Korea	Jeju-do, Seogwipo-si	33	126	20130905	20190614_5

Supplementary Table 4.1. STRUCTURE output and implementing the Evanno method of Japanese native section *Lemna* collected in this study.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	20	-22930.6	1.177095	—	—	—
2	20	-8345.45	2.144638	14585.12	12372.69	5769.127
3	20	-6133.03	2.914912	2212.425	1798.463	616.9872
4	19	-5719.06	312.9101	413.9618	248.7437	0.794936
5	20	-5553.85	371.3443	165.2182	78.12871	0.210394
6	18	-5466.76	411.6458	87.08944	303.1549	0.736446
7	19	-5682.82	438.5412	-216.065	357.8708	0.816048
8	19	-5541.02	720.3645	141.8053	349.1095	0.484629
9	15	-5748.32	368.527	-207.304	518.6909	1.407471
10	18	-5436.93	799.6286	311.3867	—	—

Supplementary Table 4.2. The Correlation of Estimates of six characters from 55 plants (eight strains) of *L. minor*, *L. japonica*, and *L. turionifera*.

	Frond number	Frond thickness	Ovary diameter	Stigma width	Stamen length	Root cap length
Frond number	1	-0.0933	0.4624	0.2428	0.0745	-0.1301
Frond thickness	-0.0933	1	0.2096	0.5397	-0.5648	0.604
Ovary diameter	0.4624	0.2096	1	0.4986	-0.1928	0.1984
Stigma width	0.2428	0.5397	0.4986	1	-0.4539	0.3895
Stamen length	0.0745	-0.5648	-0.1928	-0.4539	1	-0.4596
Root cap length	-0.1301	0.604	0.1984	0.3895	-0.4596	1

Supplementary Table 4.3. Genetic distance pairwise matrix between 39 individuals of *L. japonica*

	Strain no.	1	2	3	4	5	6
1	NGY109	0.000					
2	NGY109(4)	0.120	0.000				
3	NGY109(1)	0.075	0.066	0.000			
4	NGY109(2)	0.082	0.103	0.036	0.000		
5	NGY109(3)	0.059	0.061	0.038	0.051	0.000	
6	NGY109(1)-c1	0.098	0.147	0.059	0.064	0.067	0.000
7	NGY109(1)-c2	0.071	0.107	0.068	0.071	0.045	0.089
8	NGY109(1)-c3	0.067	0.127	0.063	0.077	0.049	0.089
9	NGY69-2	0.081	0.083	0.023	0.058	0.054	0.071
10	NGY69	0.103	0.112	0.044	0.056	0.063	0.085
11	NGY204	0.108	0.074	0.033	0.058	0.056	0.062
12	NGY204-st2	0.112	0.147	0.121	0.108	0.105	0.136
13	NGY149	0.108	0.078	0.041	0.088	0.058	0.091
14	NGY149-st2	0.096	0.065	0.044	0.078	0.054	0.090
15	NGY149-st3	0.089	0.075	0.045	0.060	0.052	0.082
16	NGY78	0.102	0.078	0.070	0.087	0.055	0.063
17	NGY55	0.094	0.116	0.061	0.074	0.072	0.095
18	NGY179	0.106	0.104	0.064	0.094	0.069	0.092
19	NGY179-2	0.063	0.076	0.045	0.082	0.052	0.078
20	NGY191-1	0.175	0.167	0.171	0.191	0.180	0.197
21	NGY191-2	0.060	0.078	0.055	0.059	0.059	0.090
22	NGY194	0.066	0.110	0.051	0.070	0.047	0.090
23	NGY194-2	0.086	0.113	0.051	0.075	0.068	0.066
24	NGY194-3	0.115	0.118	0.079	0.097	0.098	0.093
25	KHB1420047	0.187	0.171	0.150	0.192	0.173	0.189
26	S4	0.123	0.118	0.097	0.127	0.133	0.135
27	NGY188	0.097	0.114	0.096	0.097	0.089	0.098
28	NGY188-2	0.117	0.103	0.080	0.082	0.079	0.093
29	NGY198	0.088	0.080	0.064	0.078	0.059	0.092
30	NGY195	0.126	0.114	0.102	0.087	0.064	0.125
31	NGY187	0.097	0.102	0.107	0.118	0.094	0.095
32	NGY187-11	0.079	0.063	0.040	0.070	0.063	0.078
33	NGY187-12	0.086	0.067	0.058	0.074	0.048	0.092
34	NGY119	0.114	0.082	0.106	0.105	0.074	0.117
35	NGY37	0.104	0.103	0.059	0.070	0.066	0.077
36	NGY38	0.122	0.160	0.093	0.122	0.104	0.141
37	NGY207	0.134	0.167	0.100	0.146	0.131	0.142
38	NGY68	0.072	0.088	0.075	0.090	0.048	0.116
39	KHB1435284	0.128	0.122	0.097	0.133	0.108	0.149

Supplementary Table 4.3. continued

	Strain no.	7	8	9	10	11	12
1	NGY109						
2	NGY109(4)						
3	NGY109(1)						
4	NGY109(2)						
5	NGY109(3)						
6	NGY109(1)-c1						
7	NGY109(1)-c2	0.000					
8	NGY109(1)-c3	0.063	0.000				
9	NGY69-2	0.059	0.064	0.000			
10	NGY69	0.093	0.078	0.056	0.000		
11	NGY204	0.089	0.077	0.039	0.041	0.000	
12	NGY204-st2	0.089	0.133	0.075	0.121	0.129	0.000
13	NGY149	0.068	0.074	0.032	0.067	0.038	0.107
14	NGY149-st2	0.066	0.079	0.034	0.057	0.049	0.088
15	NGY149-st3	0.081	0.067	0.042	0.068	0.018	0.086
16	NGY78	0.071	0.086	0.054	0.072	0.047	0.112
17	NGY55	0.074	0.080	0.065	0.087	0.076	0.100
18	NGY179	0.084	0.068	0.058	0.096	0.088	0.145
19	NGY179-2	0.069	0.065	0.044	0.069	0.049	0.108
20	NGY191-1	0.185	0.161	0.173	0.167	0.157	0.220
21	NGY191-2	0.069	0.055	0.046	0.067	0.054	0.094
22	NGY194	0.077	0.077	0.049	0.071	0.049	0.119
23	NGY194-2	0.058	0.056	0.067	0.065	0.054	0.110
24	NGY194-3	0.086	0.106	0.071	0.087	0.090	0.119
25	KHB1420047	0.164	0.203	0.159	0.167	0.190	0.200
26	S4	0.145	0.120	0.100	0.106	0.090	0.157
27	NGY188	0.089	0.072	0.087	0.098	0.078	0.133
28	NGY188-2	0.090	0.106	0.060	0.091	0.059	0.119
29	NGY198	0.089	0.096	0.049	0.084	0.055	0.129
30	NGY195	0.080	0.089	0.072	0.099	0.081	0.113
31	NGY187	0.100	0.081	0.080	0.104	0.078	0.147
32	NGY187-11	0.093	0.081	0.035	0.045	0.043	0.144
33	NGY187-12	0.088	0.057	0.052	0.075	0.052	0.104
34	NGY119	0.091	0.102	0.098	0.120	0.082	0.135
35	NGY37	0.083	0.101	0.046	0.067	0.053	0.114
36	NGY38	0.126	0.112	0.100	0.089	0.089	0.153
37	NGY207	0.146	0.117	0.130	0.125	0.152	0.150
38	NGY68	0.065	0.089	0.057	0.092	0.065	0.113
39	KHB1435284	0.123	0.120	0.075	0.090	0.128	0.139

Supplementary Table 4.3. continued

	Strain no.	13	14	15	16	17	18
1	NGY109						
2	NGY109(4)						
3	NGY109(1)						
4	NGY109(2)						
5	NGY109(3)						
6	NGY109(1)-c1						
7	NGY109(1)-c2						
8	NGY109(1)-c3						
9	NGY69-2						
10	NGY69						
11	NGY204						
12	NGY204-st2						
13	NGY149	0.000					
14	NGY149-st2	0.053	0.000				
15	NGY149-st3	0.052	0.048	0.000			
16	NGY78	0.072	0.074	0.058	0.000		
17	NGY55	0.064	0.086	0.076	0.085	0.000	
18	NGY179	0.082	0.068	0.061	0.099	0.095	0.000
19	NGY179-2	0.048	0.054	0.060	0.070	0.082	0.066
20	NGY191-1	0.185	0.173	0.140	0.187	0.145	0.185
21	NGY191-2	0.047	0.067	0.048	0.062	0.049	0.061
22	NGY194	0.061	0.049	0.036	0.067	0.092	0.068
23	NGY194-2	0.048	0.069	0.049	0.067	0.068	0.074
24	NGY194-3	0.076	0.087	0.071	0.080	0.105	0.094
25	KHB1420047	0.140	0.179	0.181	0.176	0.227	0.176
26	S4	0.088	0.119	0.111	0.103	0.108	0.125
27	NGY188	0.092	0.101	0.068	0.074	0.091	0.079
28	NGY188-2	0.062	0.060	0.071	0.066	0.116	0.083
29	NGY198	0.071	0.062	0.068	0.074	0.091	0.100
30	NGY195	0.071	0.067	0.088	0.081	0.106	0.100
31	NGY187	0.096	0.091	0.073	0.081	0.101	0.101
32	NGY187-11	0.045	0.055	0.059	0.057	0.086	0.078
33	NGY187-12	0.078	0.080	0.038	0.071	0.066	0.078
34	NGY119	0.079	0.082	0.086	0.077	0.092	0.071
35	NGY37	0.059	0.052	0.054	0.040	0.074	0.080
36	NGY38	0.096	0.087	0.078	0.118	0.121	0.121
37	NGY207	0.123	0.139	0.146	0.149	0.170	0.152
38	NGY68	0.053	0.055	0.070	0.080	0.082	0.070
39	KHB1435284	0.088	0.093	0.090	0.103	0.135	0.120

Supplementary Table 4.3. continued

	Strain no.	19	20	21	22	23	24
1	NGY109						
2	NGY109(4)						
3	NGY109(1)						
4	NGY109(2)						
5	NGY109(3)						
6	NGY109(1)-c1						
7	NGY109(1)-c2						
8	NGY109(1)-c3						
9	NGY69-2						
10	NGY69						
11	NGY204						
12	NGY204-st2						
13	NGY149						
14	NGY149-st2						
15	NGY149-st3						
16	NGY78						
17	NGY55						
18	NGY179						
19	NGY179-2	0.000					
20	NGY191-1	0.163	0.000				
21	NGY191-2	0.052	0.159	0.000			
22	NGY194	0.068	0.182	0.072	0.000		
23	NGY194-2	0.062	0.161	0.056	0.061	0.000	
24	NGY194-3	0.075	0.220	0.079	0.089	0.102	0.000
25	KHB1420047	0.173	0.250	0.150	0.155	0.180	0.180
26	S4	0.108	0.243	0.101	0.100	0.138	0.108
27	NGY188	0.066	0.183	0.071	0.093	0.085	0.128
28	NGY188-2	0.069	0.171	0.067	0.057	0.076	0.097
29	NGY198	0.055	0.144	0.059	0.056	0.057	0.106
30	NGY195	0.089	0.210	0.081	0.082	0.107	0.141
31	NGY187	0.070	0.148	0.065	0.076	0.082	0.112
32	NGY187-11	0.054	0.173	0.054	0.062	0.069	0.074
33	NGY187-12	0.063	0.163	0.052	0.082	0.045	0.094
34	NGY119	0.077	0.203	0.073	0.089	0.094	0.093
35	NGY37	0.053	0.188	0.052	0.071	0.074	0.090
36	NGY38	0.096	0.218	0.098	0.091	0.099	0.151
37	NGY207	0.146	0.250	0.122	0.130	0.139	0.144
38	NGY68	0.062	0.173	0.062	0.077	0.079	0.093
39	KHB1435284	0.103	0.199	0.082	0.122	0.097	0.110

Supplementary Table 4.3. continued

	Strain no.	25	26	27	28	29	30
1	NGY109						
2	NGY109(4)						
3	NGY109(1)						
4	NGY109(2)						
5	NGY109(3)						
6	NGY109(1)-c1						
7	NGY109(1)-c2						
8	NGY109(1)-c3						
9	NGY69-2						
10	NGY69						
11	NGY204						
12	NGY204-st2						
13	NGY149						
14	NGY149-st2						
15	NGY149-st3						
16	NGY78						
17	NGY55						
18	NGY179						
19	NGY179-2						
20	NGY191-1						
21	NGY191-2						
22	NGY194						
23	NGY194-2						
24	NGY194-3						
25	KHB1420047	0.000					
26	S4	0.185	0.000				
27	NGY188	0.191	0.120	0.000			
28	NGY188-2	0.173	0.116	0.100	0.000		
29	NGY198	0.178	0.099	0.074	0.085	0.000	
30	NGY195	0.134	0.119	0.082	0.077	0.093	0.000
31	NGY187	0.196	0.118	0.076	0.087	0.083	0.100
32	NGY187-11	0.181	0.085	0.087	0.069	0.048	0.102
33	NGY187-12	0.186	0.108	0.058	0.092	0.080	0.099
34	NGY119	0.191	0.147	0.108	0.108	0.086	0.111
35	NGY37	0.152	0.076	0.067	0.077	0.069	0.086
36	NGY38	0.183	0.144	0.108	0.124	0.097	0.114
37	NGY207	0.257	0.167	0.131	0.182	0.144	0.151
38	NGY68	0.159	0.115	0.091	0.094	0.084	0.075
39	KHB1435284	0.191	0.149	0.139	0.114	0.115	0.104

Supplementary Table 4.3. continued

	Strain no.	31	32	33	34	35	36
1	NGY109						
2	NGY109(4)						
3	NGY109(1)						
4	NGY109(2)						
5	NGY109(3)						
6	NGY109(1)-c1						
7	NGY109(1)-c2						
8	NGY109(1)-c3						
9	NGY69-2						
10	NGY69						
11	NGY204						
12	NGY204-st2						
13	NGY149						
14	NGY149-st2						
15	NGY149-st3						
16	NGY78						
17	NGY55						
18	NGY179						
19	NGY179-2						
20	NGY191-1						
21	NGY191-2						
22	NGY194						
23	NGY194-2						
24	NGY194-3						
25	KHB1420047						
26	S4						
27	NGY188						
28	NGY188-2						
29	NGY198						
30	NGY195						
31	NGY187	0.000					
32	NGY187-11	0.087	0.000				
33	NGY187-12	0.076	0.048	0.000			
34	NGY119	0.095	0.102	0.085	0.000		
35	NGY37	0.078	0.053	0.070	0.101	0.000	
36	NGY38	0.129	0.088	0.102	0.143	0.108	0.000
37	NGY207	0.148	0.117	0.131	0.173	0.147	0.156
38	NGY68	0.079	0.069	0.062	0.089	0.085	0.097
39	KHB1435284	0.114	0.125	0.114	0.099	0.116	0.162

Supplementary Table 4.3. continued

	Strain no.	37	38	39
1	NGY109			
2	NGY109(4)			
3	NGY109(1)			
4	NGY109(2)			
5	NGY109(3)			
6	NGY109(1)-c1			
7	NGY109(1)-c2			
8	NGY109(1)-c3			
9	NGY69-2			
10	NGY69			
11	NGY204			
12	NGY204-st2			
13	NGY149			
14	NGY149-st2			
15	NGY149-st3			
16	NGY78			
17	NGY55			
18	NGY179			
19	NGY179-2			
20	NGY191-1			
21	NGY191-2			
22	NGY194			
23	NGY194-2			
24	NGY194-3			
25	KHB1420047			
26	S4			
27	NGY188			
28	NGY188-2			
29	NGY198			
30	NGY195			
31	NGY187			
32	NGY187-11			
33	NGY187-12			
34	NGY119			
35	NGY37			
36	NGY38			
37	NGY207	0.000		
38	NGY68	0.146	0.000	
39	KHB1435284	0.183	0.104	0.000

Supplementary Table 4.4. Prior distributions of the parameters used in DIYABC.

Parameter	Probability distribution	Minimum	Maximum
Effective population size			
N1	uniform	10	12,000
N2	uniform	10	15,000
N3	uniform	10	8,000
Na	uniform	10	10,000
Time scale in generations			
t1	uniform	10	15,000
t2	uniform	10	40,000
t3	uniform	10	40,000

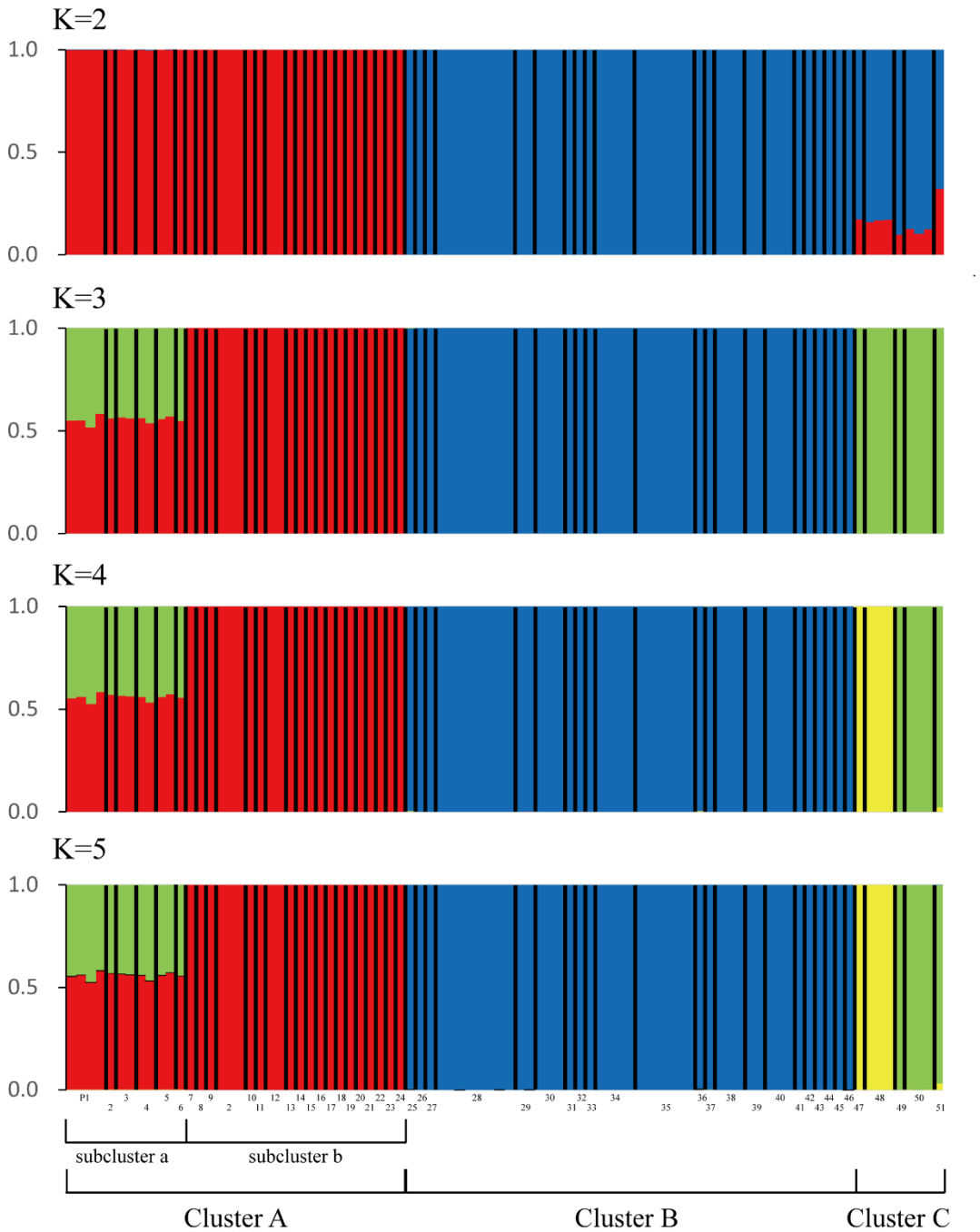


Figure 4.1. Structure analysis of K=2-5 based on 395 SNP loci of Japanese section *Lemna*. Population codes were written under each bar plot.

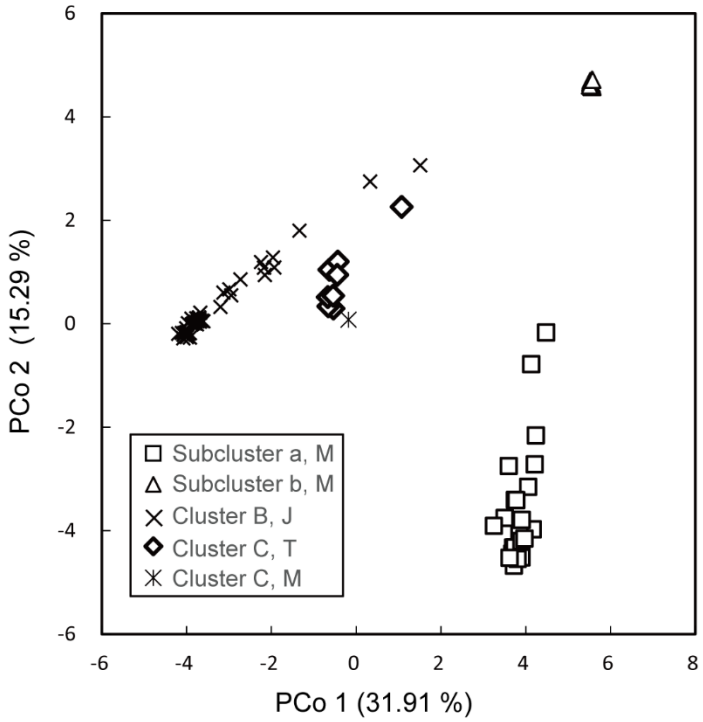


Figure 4.2. Results of principal-coordinates analysis (PCoA) based on 395 SNPs data among 88 plants of Japanese sect. *Lemna*. The cluster which sample belongs to and the samples' haplotype were provided. Proportion of total variance of each axis was also shown.

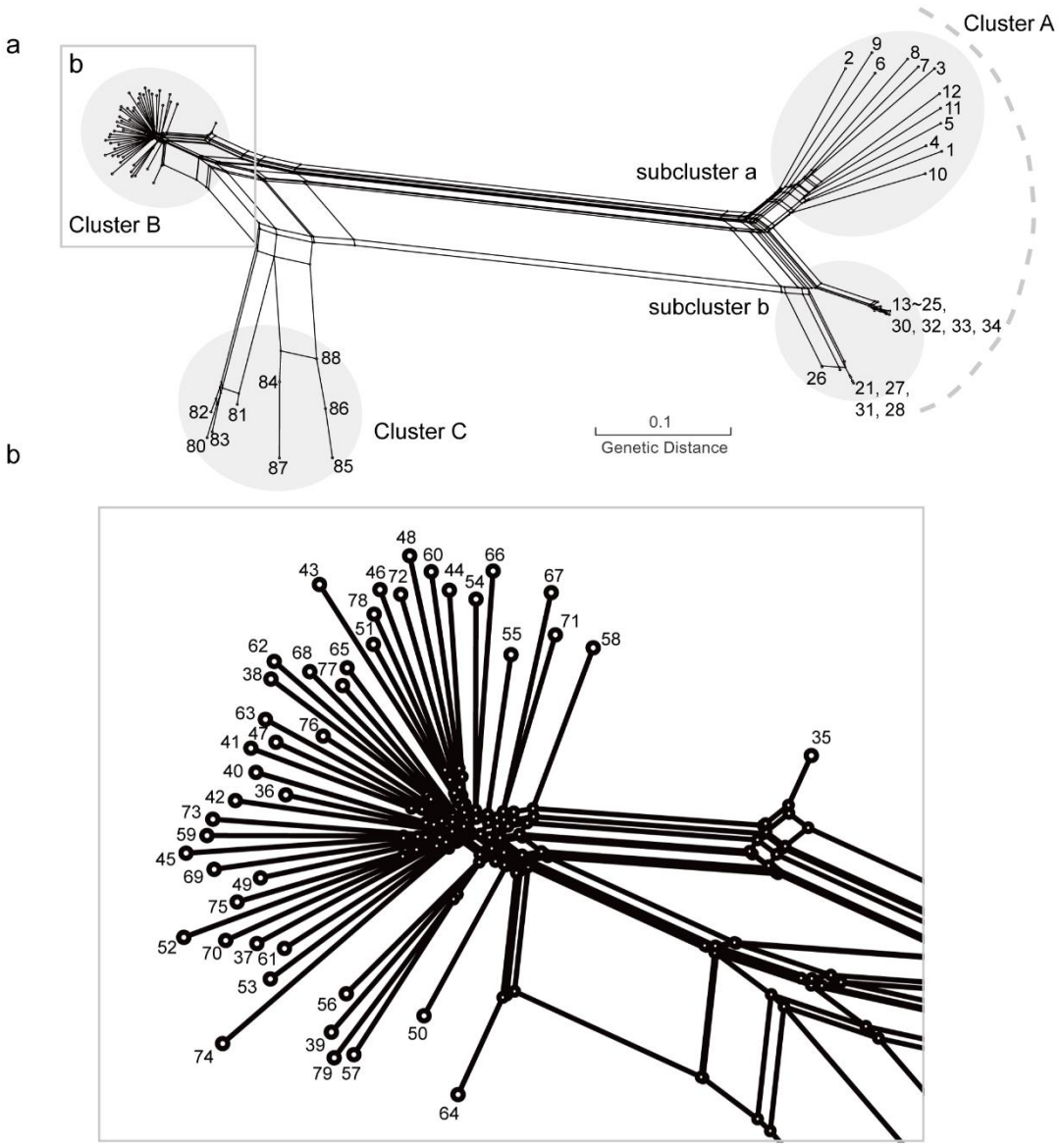


Figure 4.3. Neighbor-net Network based on 395 SNPs data among 88 plants of Japanese sect. *Lemna*. Each number means sample number that was indicated in Table 4.1.

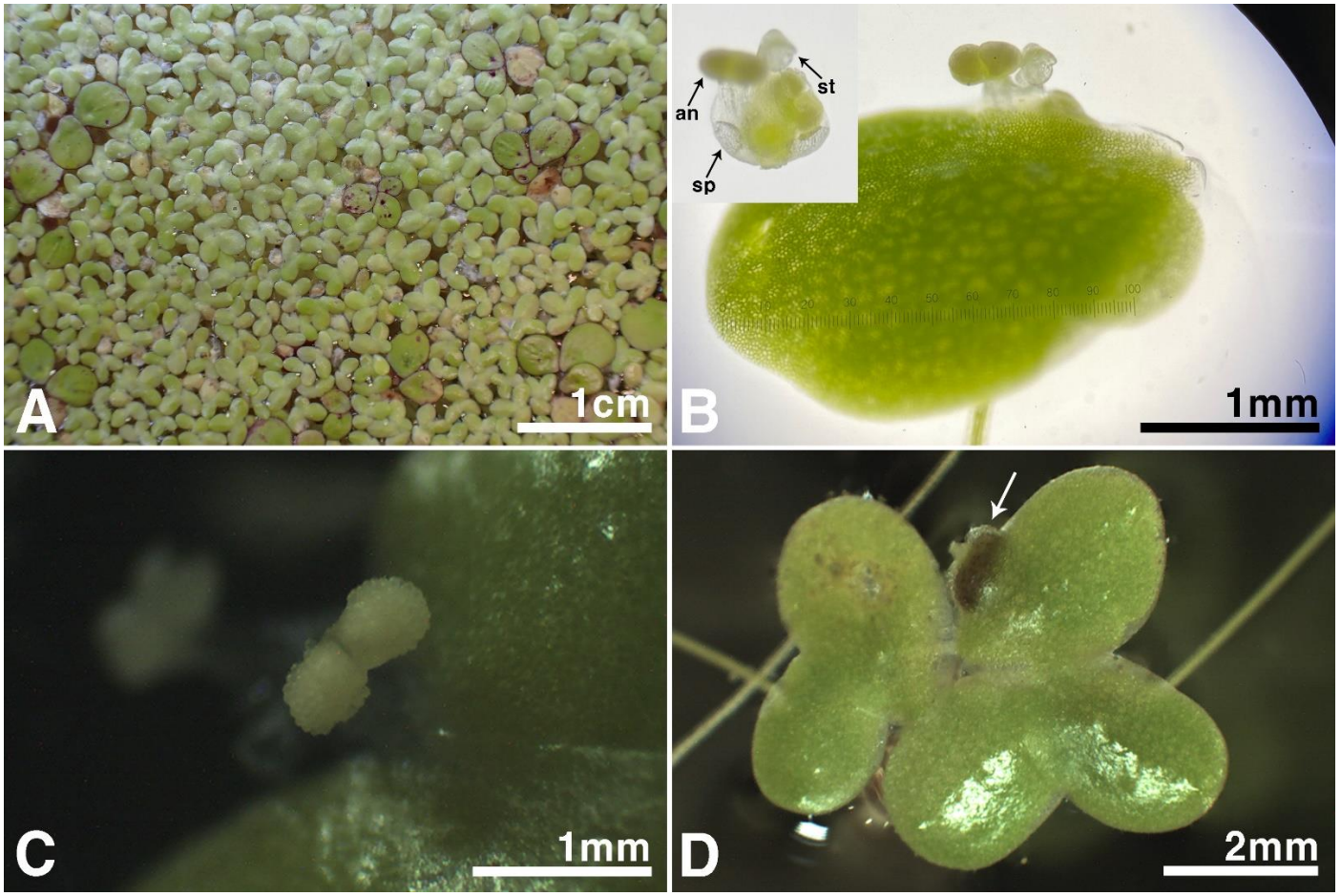


Figure 4.4. The morphology of Japanese *Lemna minor* L. A, Flowering plants in their native habitat (2021. Jun., Joetsu-si, Niigata Pref.) [The typically big, rounded fronds are *Spirodela polyrhiza* (L.) Schleid.]. B, A flowering whole plant of strain NGY173-11. Flower which is surrounded by transparent spathe; Pistil starts maturing earlier than two stamens but their maturing time is not completely different (an, anther; sp, spathe; st, stigma) C, Pollen grains in opened anthers of NGY152-11 on 50ml of 0.8% agar-including 20^{-1} NH_4^+ free Hutner's medium in 90×20 mm sized petri-dish. D, Fruiting plant found in the place where photo A was taken; The fruit margin is winged (arrow).

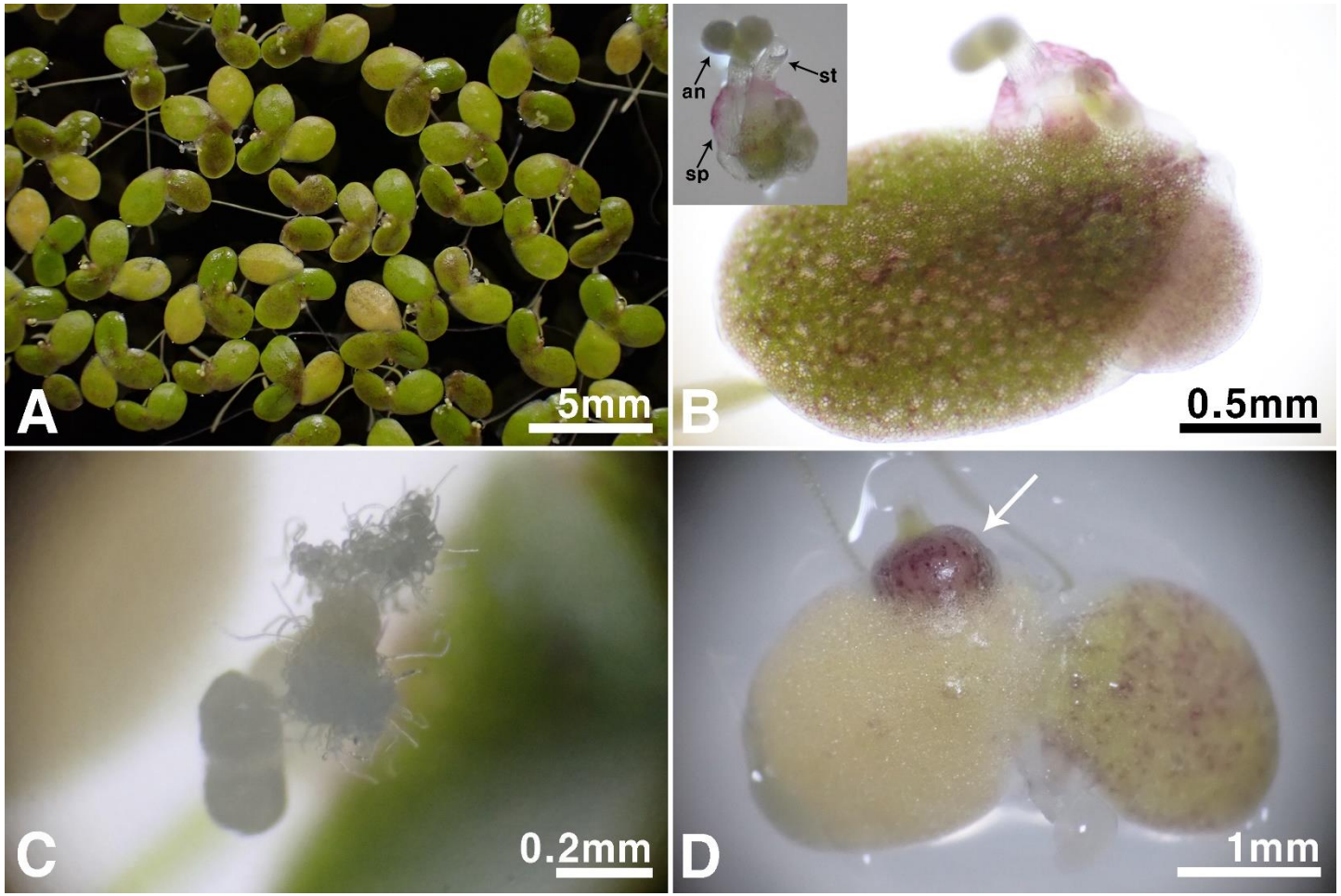


Figure 4.5. The morphology of Japanese *Lemna turionifera* Landolt. A, Flowering whole plants of strain NGY200-13. B, A flowering whole plant of strain NGY200-13. Flower which is surrounded by purple-pigmented spathe; Pistil starts maturing earlier than two stamens but their maturing time is not completely different (an, anther; sp, spathe; st, stigma) C, Germinated pollen grains on opened anthers of NGY151-11 on 50ml of 0.8% agar-including 1/20 NH_4^+ free Hutner's medium in 90×20 mm sized petri-dish. D, Fruit of strain NGY200-13; The fruit margin is not winged (arrow).

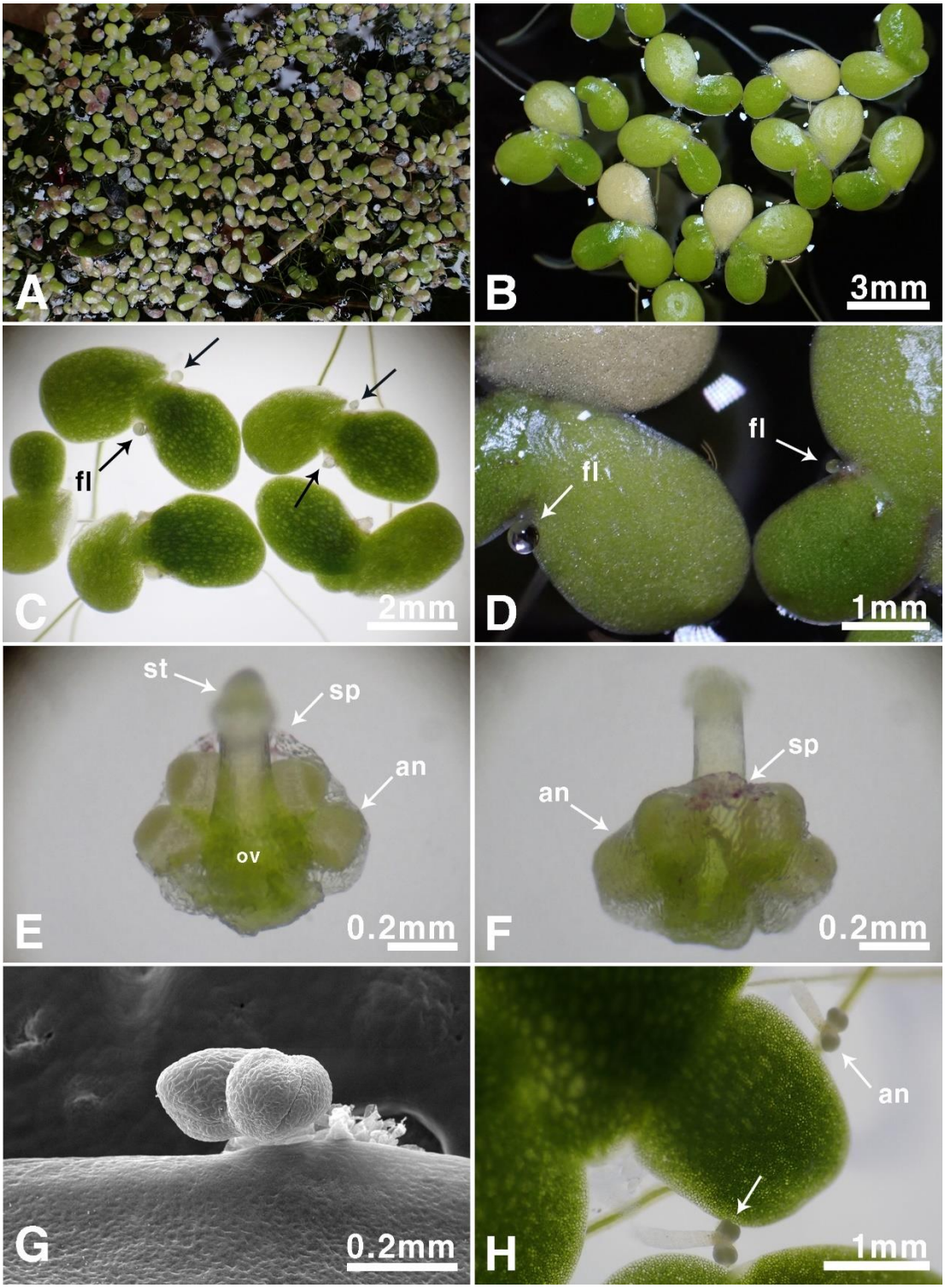


Figure 4.6. The morphology of Japanese *Lemna japonica* Landolt. A, plants in their native habitat (2020. Sep., Shimane); B, flower-induced strain 7182; C, strain NGY109-2 which bears two flowers in one frond (black arrows); D, flowering strain 7182, pistil is developed, and water-droplet is on the stigma (left side arrow); E, Flower's front side of strain NGY109-2 which is surrounded by pale-purple-pigmented spathe; Pistil matures earlier than two stamens (an, anther; sp, spathe; st, stigma; ov, ovary); F, Flower's back side of strain NGY109-2; Top of spathe is little pigmented. G, Stamens grow after the pistil withered, but anthers never open (strain NGY109-2, photo by HJ Jo in Andong National University, Korea). H, Stamens (white arrows) pushed out from mother fronds by new daughter fronds.

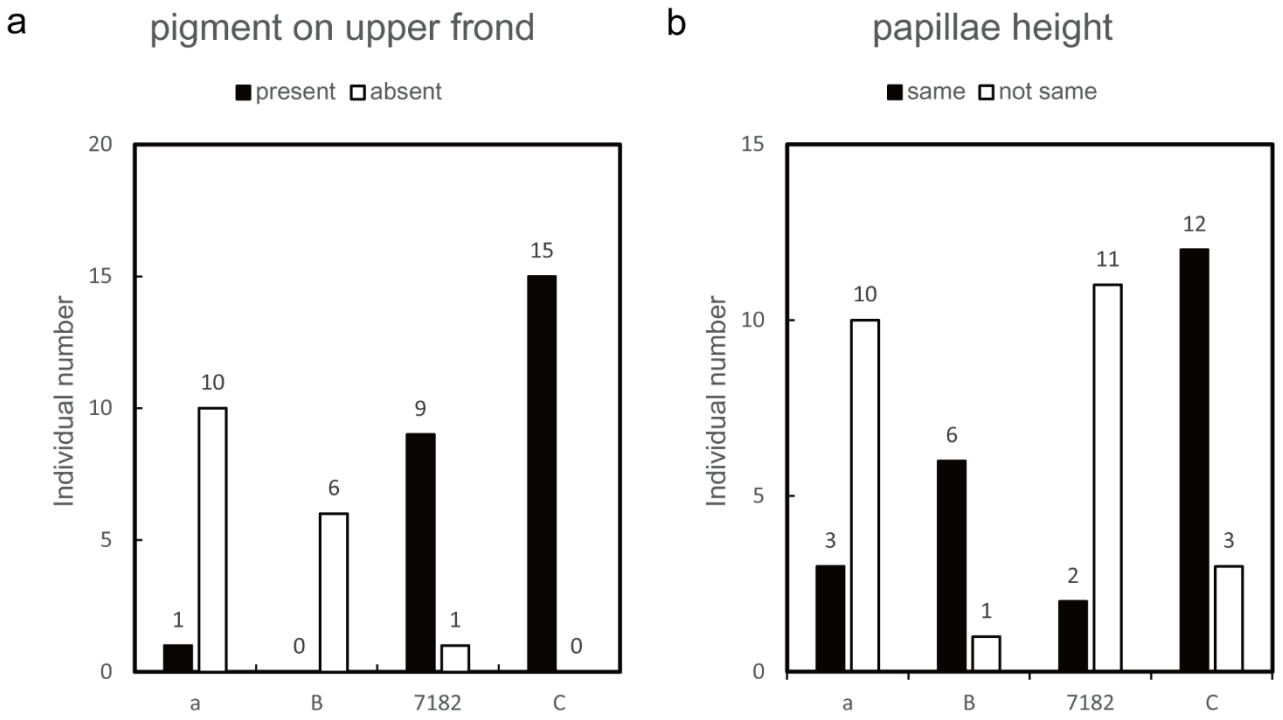


Figure 4.7. The qualitative character of of each cluster in Japanese section *Lemna*. The abbreviation a indicates subcluster a, B indicates cluster B, 7182 indicates L. japonica type strain no.7182, C indicates cluster C. (a) individual number whose frond has pigment, (b) individual number whose papillae height are same or not same.

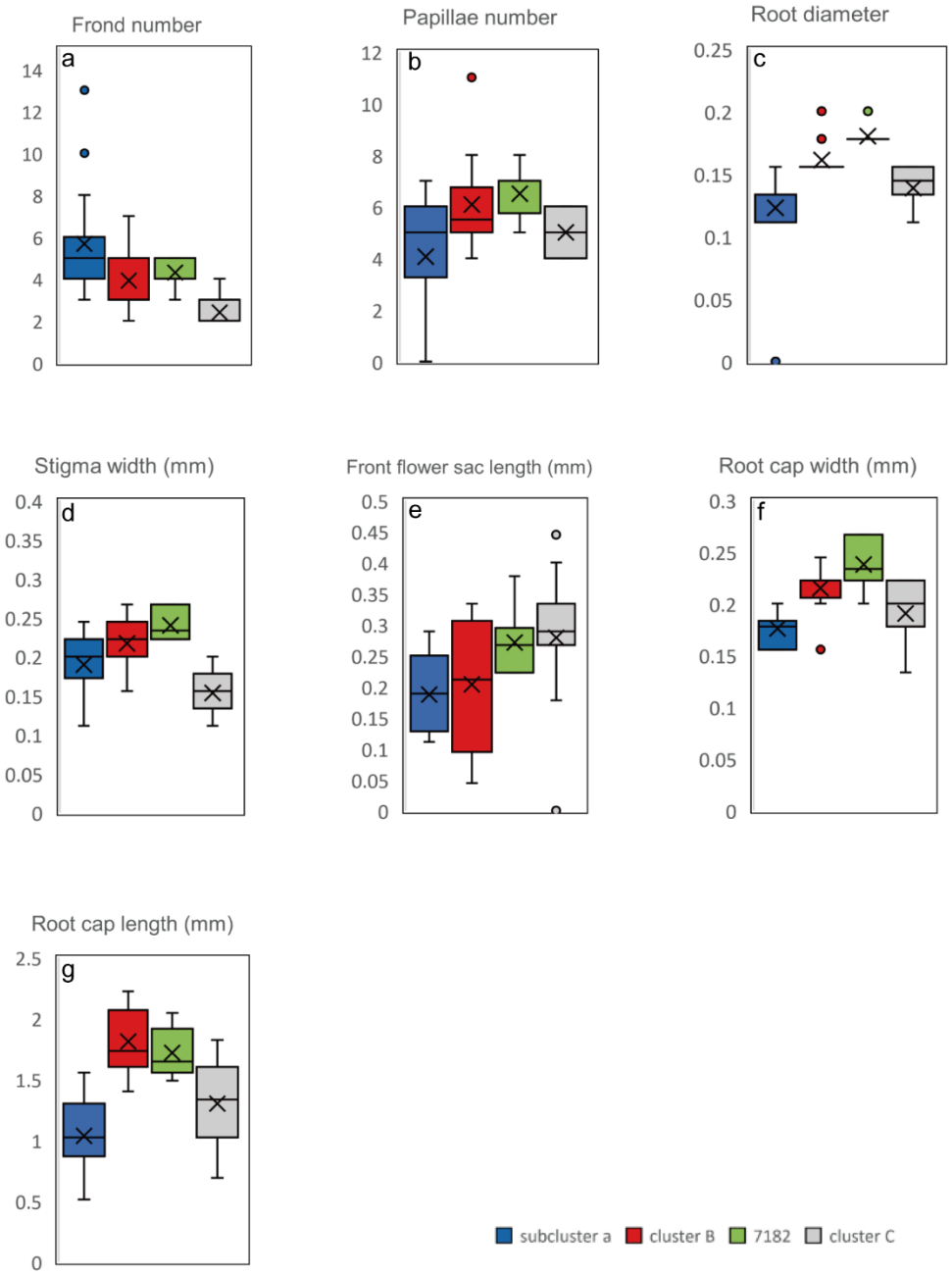


Figure 4.8. Seven morphological characters of each cluster in Japanese section *Lemna*. **a** frond number, **b** papillae number, **c** root diameter, **d** stigma width, **e** front spathe of flower length, **f** root cap width, **g** root cap length. For each box plot, top bar indicates maximum value, top end of the box indicates third quartile, the middle bar indicates median value, and the circles indicate possible outliers.

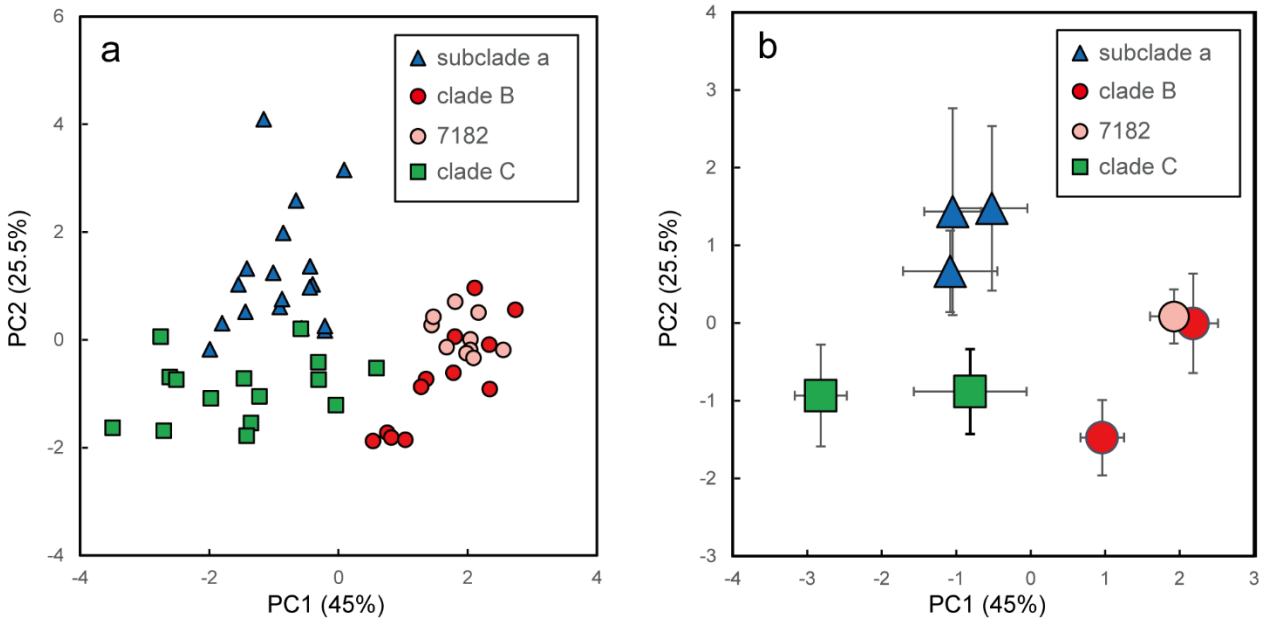


Figure 4.9. Principal Component Analysis (PCA) based on six morphological characters of each cluster in Japanese section *Lemna*. The abbreviation a indicates subcluster a, B indicates cluster B, 7182 indicates L. japonica type strain no.7182, C indicates cluster C. **a** The Principal components of 55 plants of three species in Japanese section *Lemna*. **b** The principal components averaged by strain. Error bars represent the standard error.

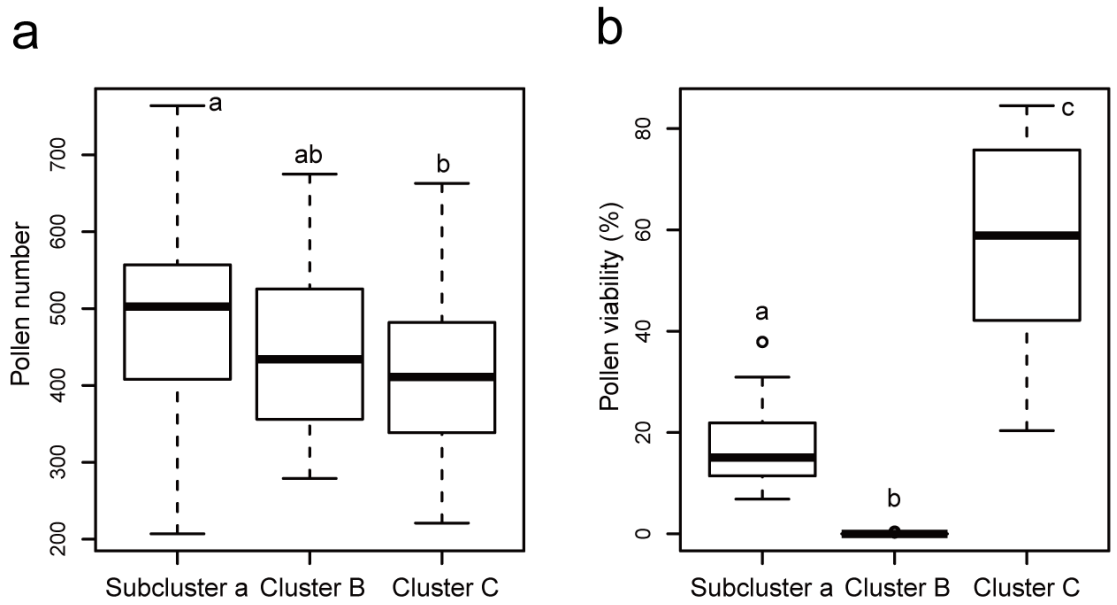
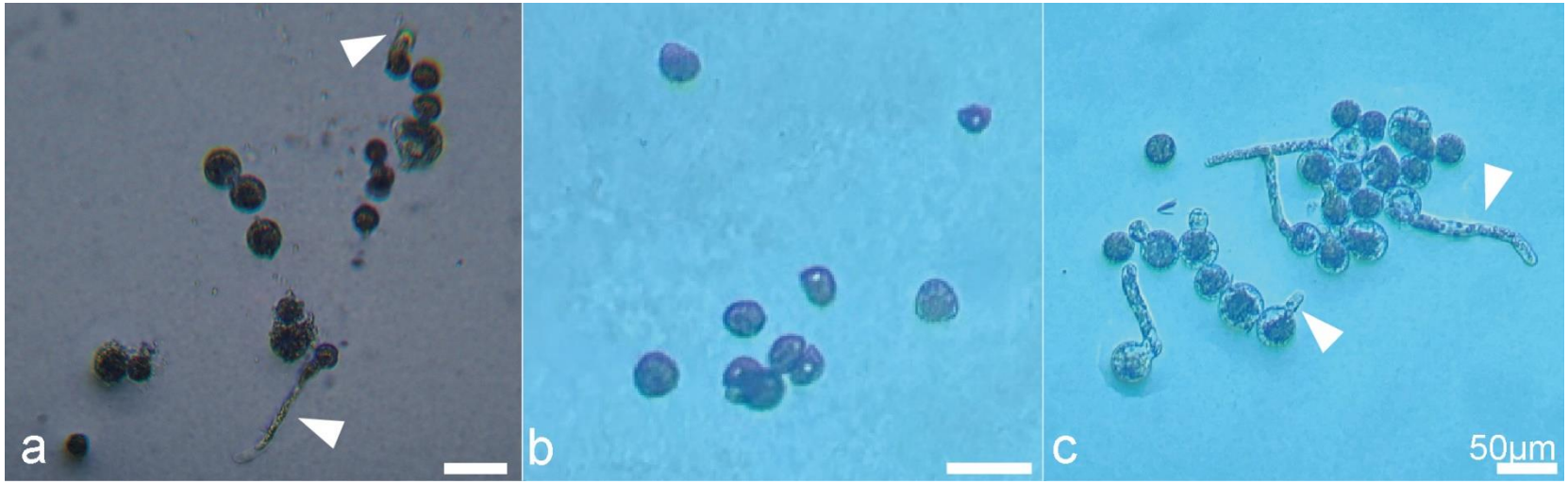


Figure 4.10. (a) Pollen number and (b) pollen viability of each cluster in Japanese section *Lemna*. For each box plot, top bar indicates maximum value, top end of the box indicates third quartile, the middle bar indicates median value, and the circles indicate possible outliers.



4.11. Germination test of pollen grains from three species in Japanese section *Lemna*. (a) *L. minor* (NGY152-11), (b) *L. japonica* (NGY187-12), (c) *L. turionifera* (NGY151). The white arrows indicate germinated pollen grains. Each scale bar indicates 50μm.

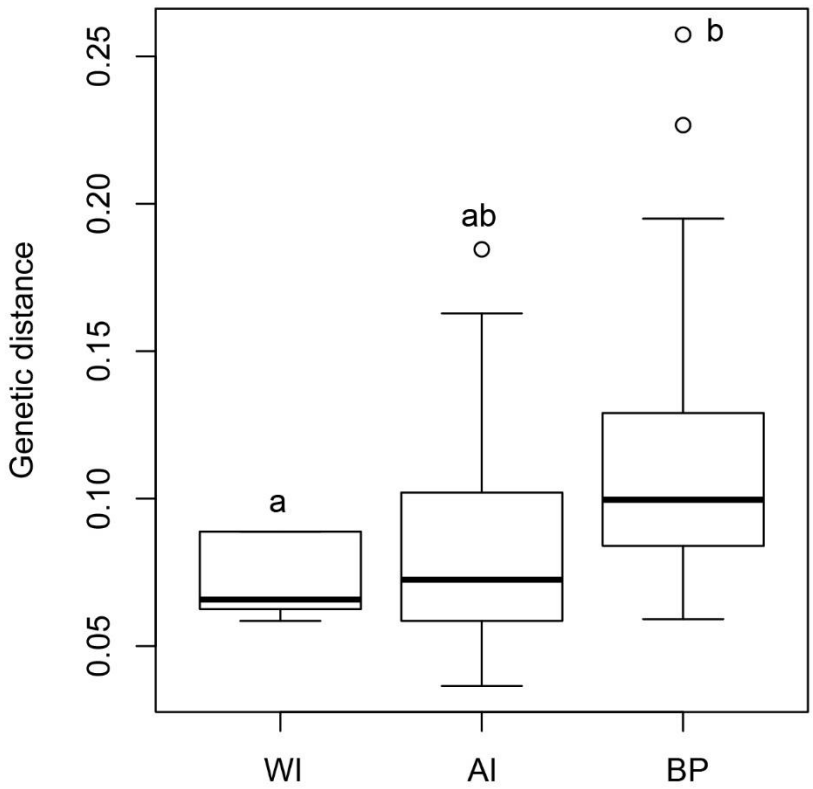


Figure 4.12. Boxplot of genetic distance pair under three conditions; WI, within individual; AI, among individuals in same population; BP, between populations. Same letters above the boxplot indicate that they are not significantly different ($p > 0.05$) by Student's test and Welch's test. For each box plot, top bar indicates maximum value, top end of the box indicates third quartile, the middle bar indicates median value, and the circles indicate possible outliers.

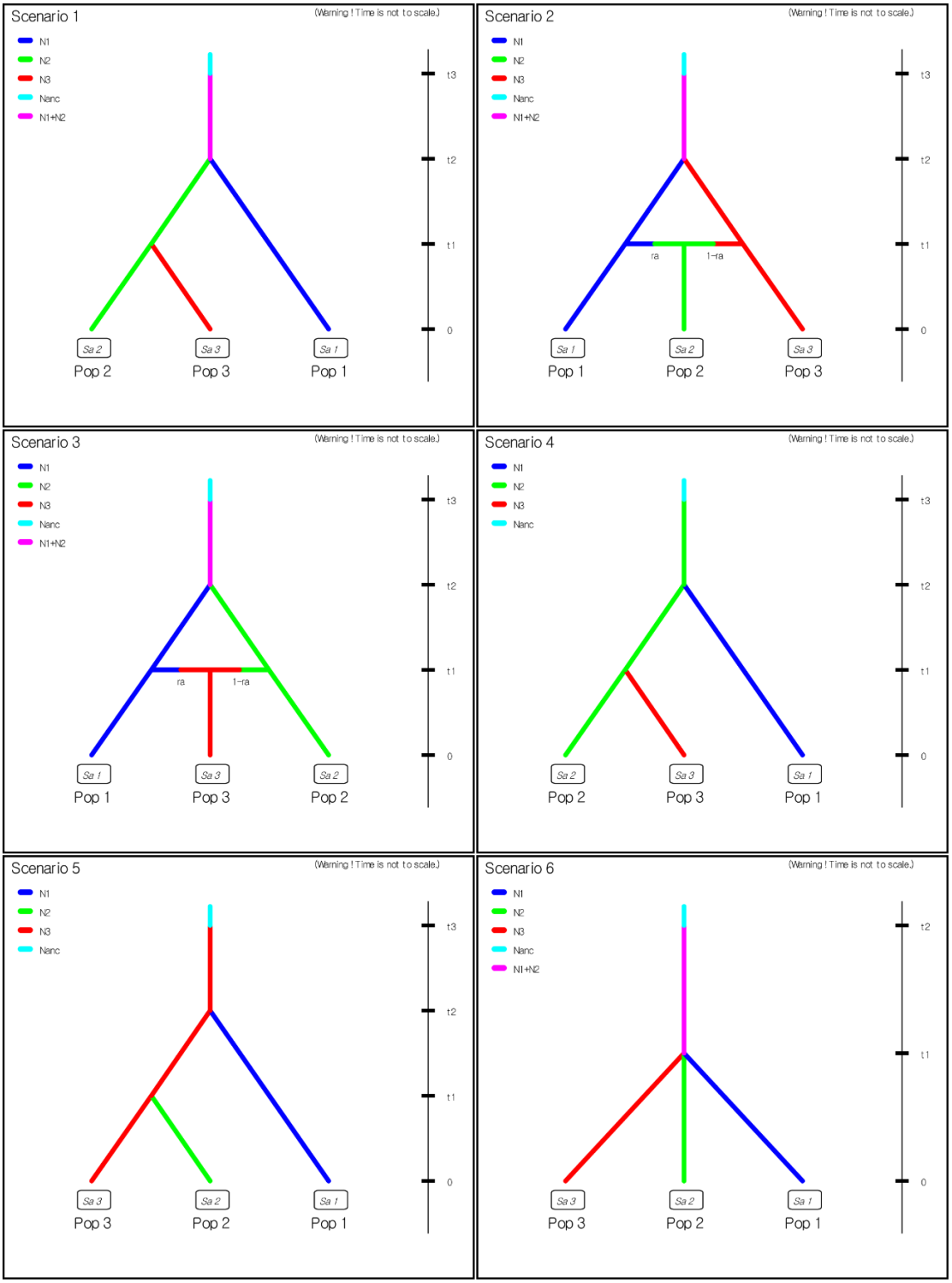


Figure 4.13. Final candidate six scenarios for Approximate Bayesian computation.

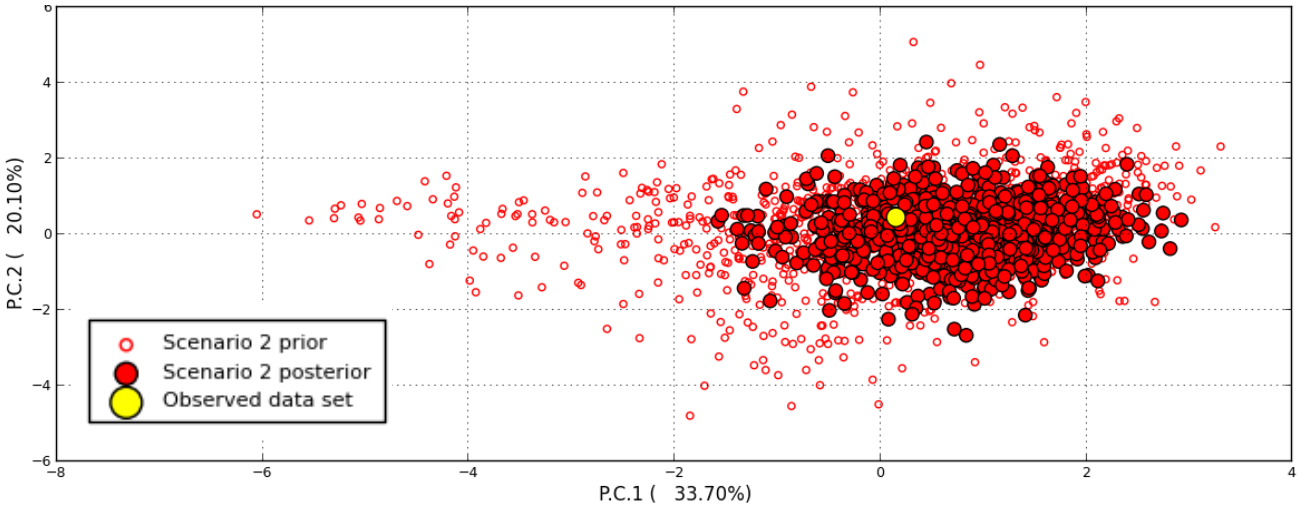


Figure 4.14. The result of PCA from data set on DIYABC model checking option.

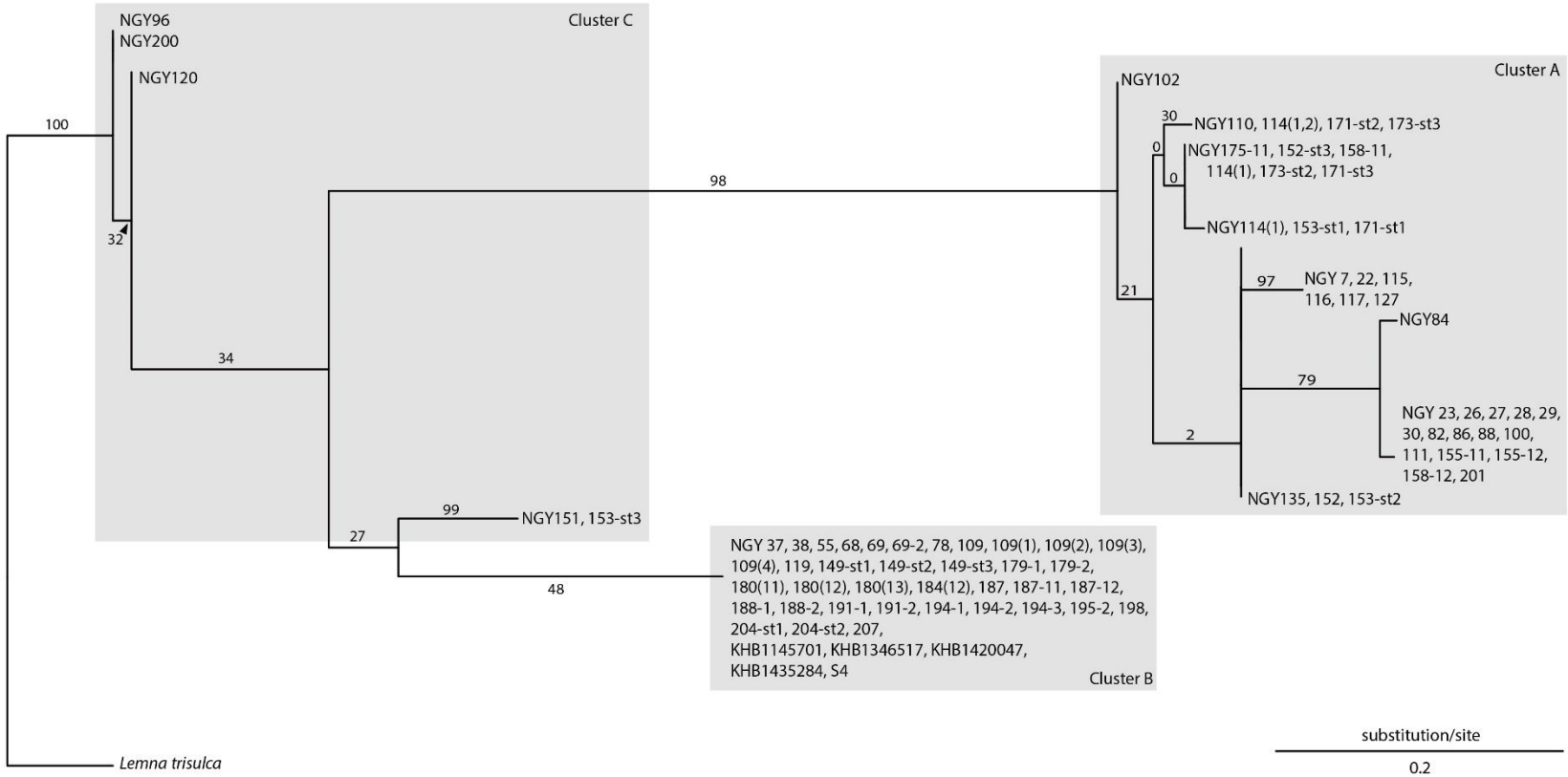


Figure 4.15. Maximum likelihood tree based on 476 SNPs data of three species in Japanese section *Lemna* with *L. trisulca*. Bootstrap values shown above the branches.

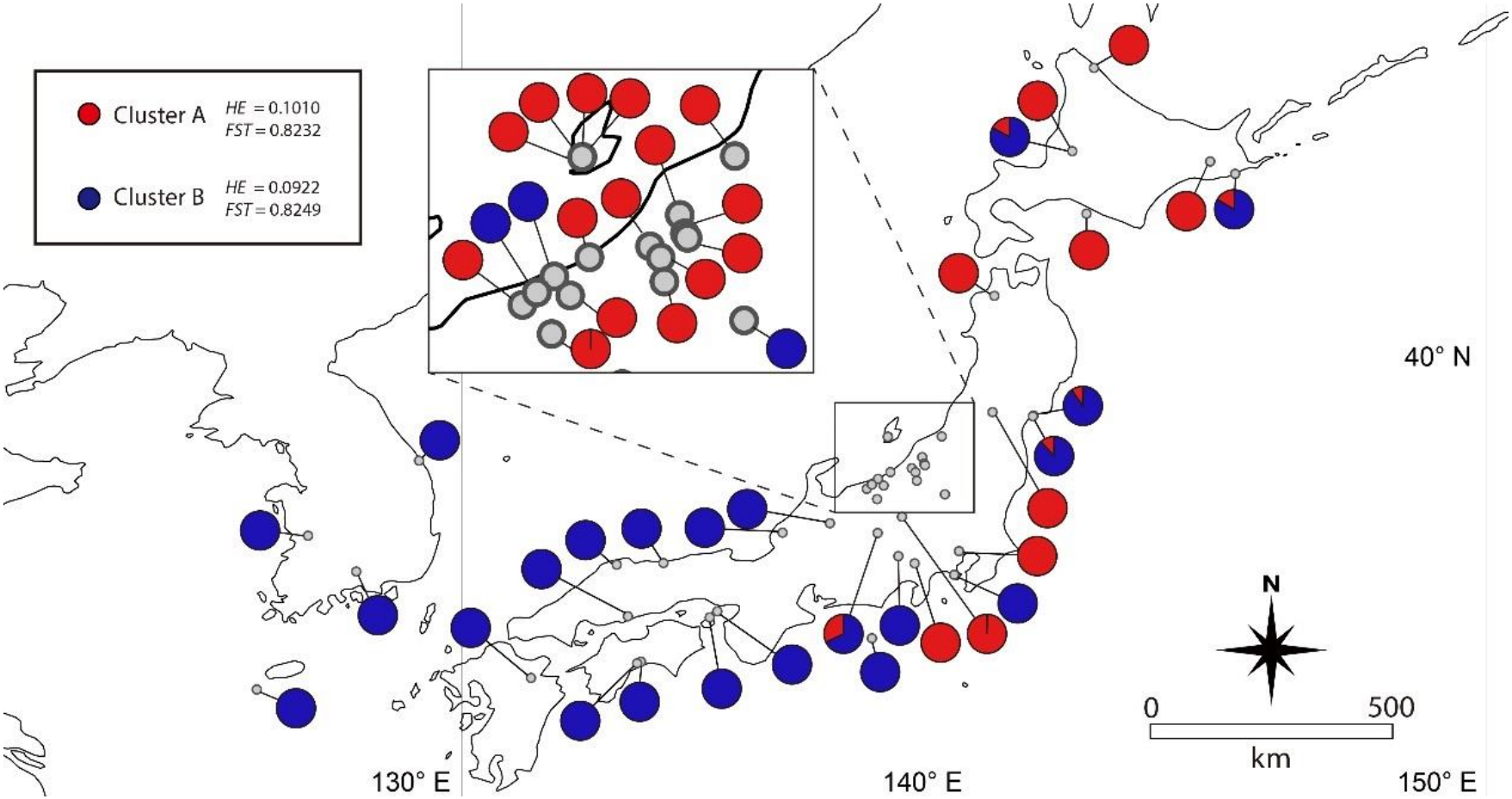


Figure 4.16. Distribution map of 88 plants of Japanese sect. *Lemna* based on 395 SNPs data in STRUCTURE analysis of K=2.

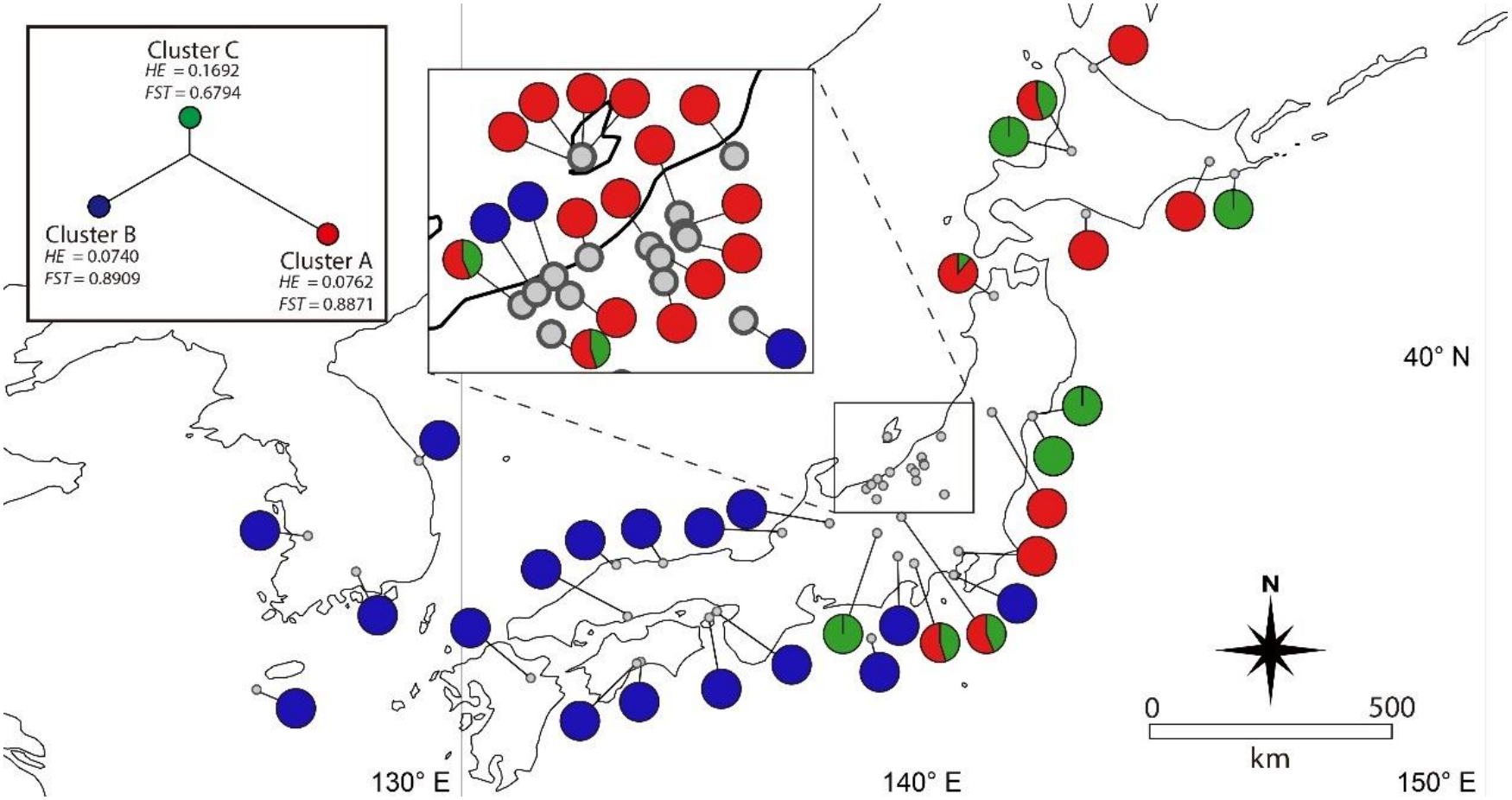
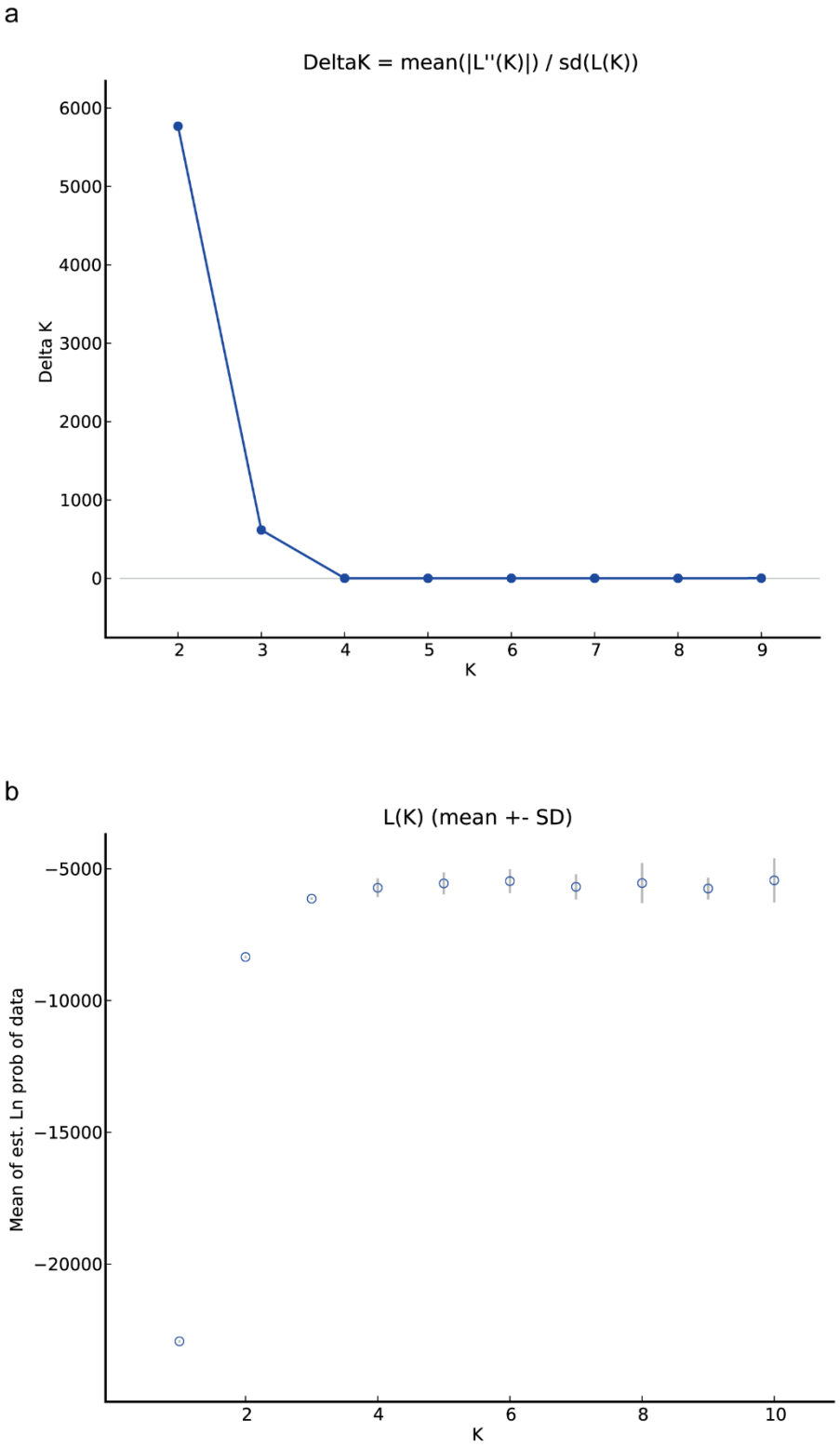
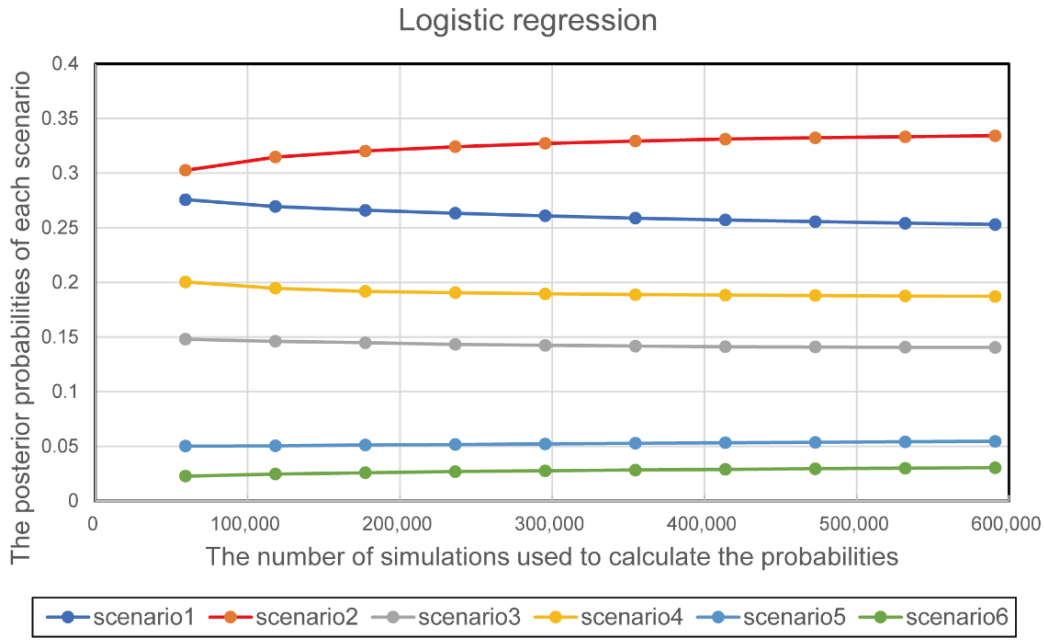


Figure 4.17. Distribution map of 88 plants of Japanese sect. *Lemna* based on 395 SNPs data in STRUCTURE analysis of K=3.



Supplementary Figure 4.1. Results of STRUCTURE analysis. (a) Delta *K* values and (b) L(K) (mean± SD) of STRUCTURE analysis in three Japanese section *Lemna* species, *L. minor*, *L. japonica*, *L. turionifera*.



Supplementary Figure 4.2. Result of the DIYABC analysis Model comparison using the logistic regression approach.

Table 5.1. Comparison of characteristics of Korean *L. turionifera* and its related species. The characters of *L. japonica* and *L. minor* were observed based on Japanese samples.

Character	*L. aequinoctialis*	*L. japonica*	*L. minor*	*L. turionifera*
Habit	Annual	Annual or perennial	Annual or perennial	Annual or perennial
Frond morphology	Widely ovate to lance-ovate	Obovate to elliptic	Obovate to elliptic	Obovate to elliptic
Cohering frond number	4 ± 2 (2–13)	4 ± 1 (2–7)	6 ± 3 (3–13)	2 ± 1.3 (1–6)
Similarity of papillae size on mid vein	Typically big on the node	Often same	Usually different	Usually same
Papillae number	3 ± 1 (2–5)	6 ± 2 (4–11)	4 ± 2 (0–7)	6 ± 1.3 (4–9)
Frond length (mm)	3.4 ± 1.0 (2.3–6.6)	3.5 ± 0.2 (3.3–4)	3.4 ± 0.6 (2.6–4.9)	2.9 ± 0.2 (2.7–3.4)
Frond width (mm)	2.4 ± 0.8 (1.5–4.3)	2.3 ± 0.1 (2.1–2.6)	2.3 ± 0.5 (1.6–3.6)	2.0 ± 0.1 (1.8–2.3)
Frond length/width	1.4 ± 0.1 (1.2–1.7)	1.5 ± 0.1 (1.3–1.6)	1.5 ± 0.1 (1.3–1.7)	1.5 ± 0.1 (1.4–1.8)
Frond upper surface	Green or pale green	Green or purplish	Green or pale green	Green or purplish
Frond lower surface	Green or pale green	Green or purplish	Green or pale green	Green or purplish
Flower organs mature time	Homogamous	Protogynous	Protogynous	Protogynous
Flower spathe color	Not colored	Pale reddish purple	Not colored	Reddish purple
Turion	Absent	Present	Absent	Present
Root sheath	Winged	Not winged	Not winged	Not winged
Root base color	Not colored	Usually purple	Not colored	Usually purple
Root tip	Acute	Obtuse	Obtuse	Obtuse
Fruit morphology	Not winged	Unknown	Winged	Not winged (not seen in Korea)

Table 5.2. Identification of *Lemna* species based on *atpF–H* intergenic spacer sequences. The sequence matching ratio (*) for our sample (NGY403-1) are also shown.

Species	Strain no.	Country	Accession no.	Reference	Sequence matching (%)*
Lemna sp.	NGY403-1	Republic of Korea	LC738859	this study	-
L. turionifera	6573	USA	MG775403	Bog et al. (2019)	100
	7683	Republic of Korea	MG775404	Bog et al. (2019)	100
	9434	Russia	MG775405	Bog et al. (2019)	100
	8339	China	GU454239	Wang et al. (2010)	99.8
	8760	Czech Republic	GU454240	Wang et al. (2010)	99.8
L. minor	9417	Germany	GU454231	Wang et al. (2010)	97.2
	7018	Turkey	GU454226	Wang et al. (2010)	97.1
	7136	USA	GU454227	Wang et al. (2010)	97.1
	7210	South Africa	GU454228	Wang et al. (2010)	97.1
	9253	Finland	GU454230	Wang et al. (2010)	96.9
L. japonica	7182	Japan	GU454225	Wang et al. (2010)	96.9
L. obscura	8896	Ecuador	MG775395	Bog et al. (2019)	96.5
	9235	Ecuador	MG775396	Bog et al. (2019)	96.5

Table 5.3. The strains and the information which were observed in this study.

Species	Strain name	Observed ramet number	Locality	Latitude & Longitude
L. aequinoctialis	NGY14	25	Goseong-gun, Gangwon-do, Republic of Korea	38.49250, 128.41722
L. japonica	NGY109-2	6	Joetsu-shi, Niigata Prefecture, Japan	37.08, 138.15
	NGY187-12	6	Sumoto-shi, Hyogo Prefecture, Japan	34.40694, 134.83667
L. minor	NGY153-14	6	Sunagawa-shi, Hokkaido Prefecture, Japan	43.50413, 141.90750
	NGY173-11	6	Nagano-shi, Nagano Prefecture, Japan	36.71467, 138.10180
	NGY114-1	6	Itoigawa-shi, Niigata Prefecture, Japan	36.91449, 137.90116
L. turionifera	NGY403-1	15	Andong-si, Gyeongsangbuk-do, Republic of Korea	36.55533, 128.74611

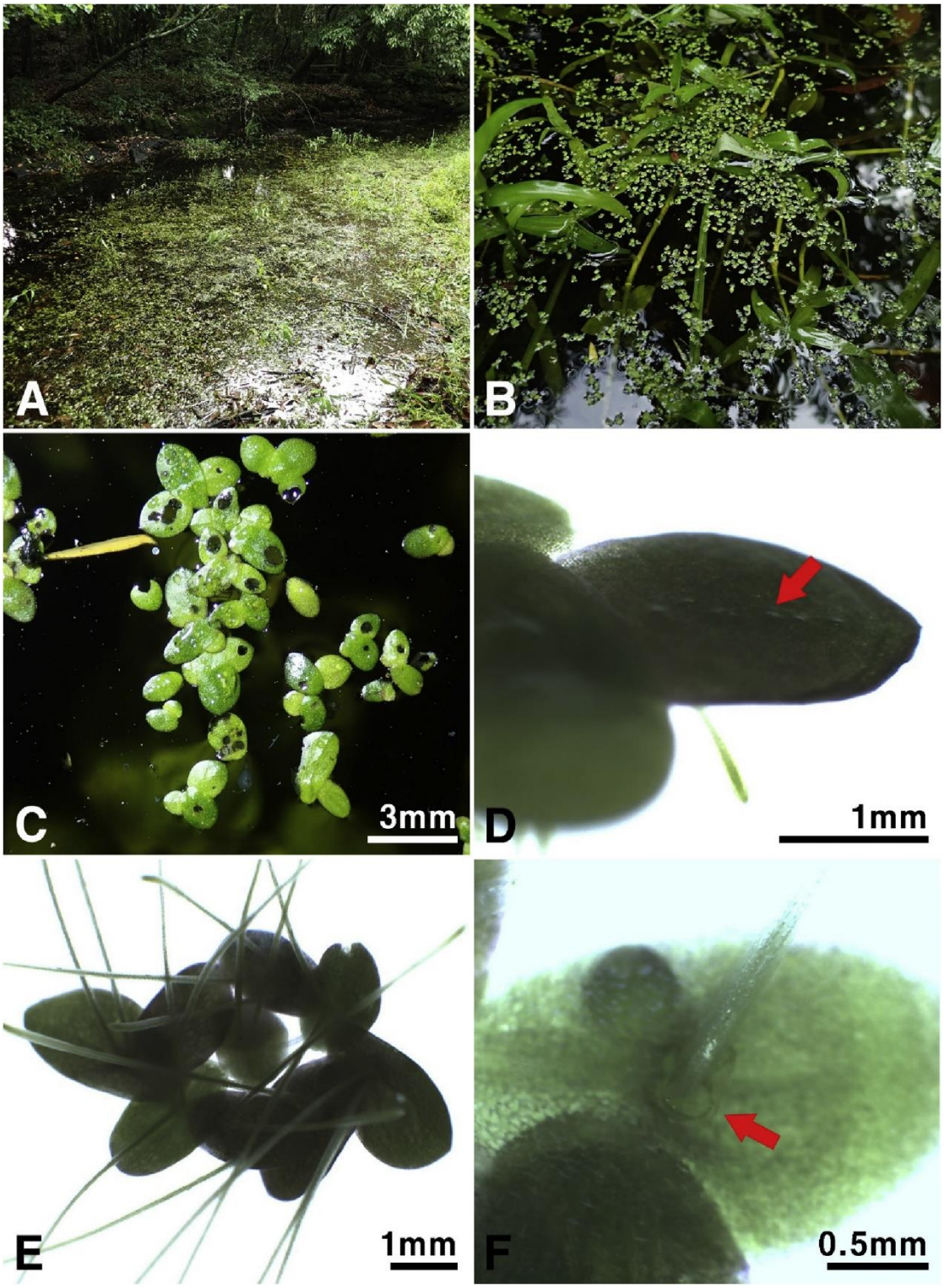


Figure 5.1. Habitat and key characteristics of Korean *Landoltia punctata*: A-C, habitat of Seonheul-ri; D, papillae on upper surface of fronds (red arrow); E, roots and lower surface of frond; F, prophyllum (red arrow).

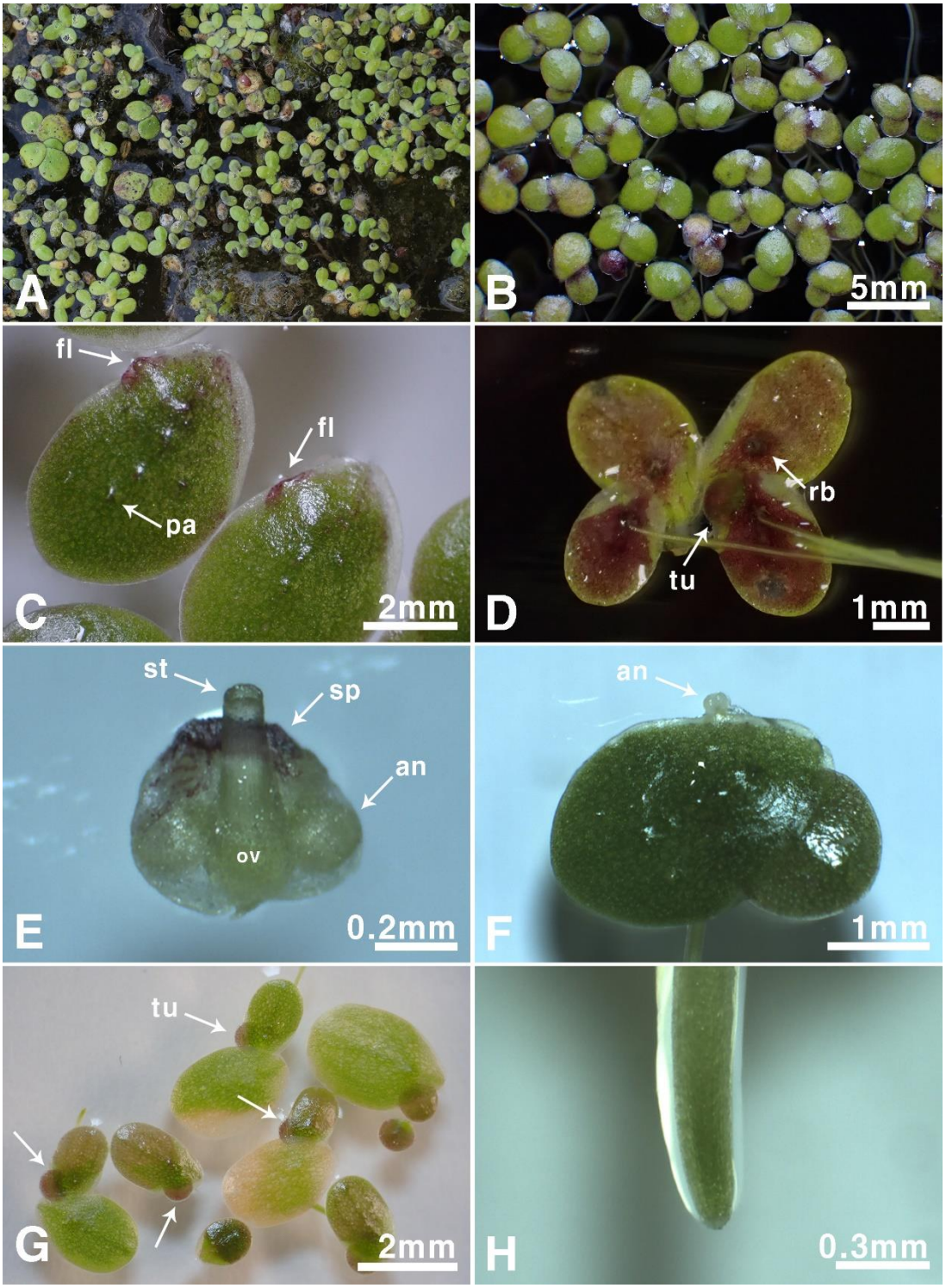


Figure 5.2. Key characteristics of Korean *Lemna turionifera* Landolt. Except for A and D, photos were taken under axenic conditions: A, plants in their native habitat [The typically big, rounded fronds are *Spirodela polyrhiza* (L.) Schleid.]; B, strain number NGY403-1 cultivated in axenic conditions in Hutner's medium; C, flowering *L. turionifera*, regular sized papillae are along the midvein (fl, flower; pa, papilla); D, purplish lower side of frond; Root base is usually purplish (rb, root base); E, flower surrounded by purplish spathe; Pistil matures earlier than two anthers (an, anther; sp, spathe; st, stigma; ov, ovary); F, flowering *L. turionifera*; Stamens mature after pistil starts to wilt; G, turion-bearing fronds (tu: turion); H. Obtuse root tip.

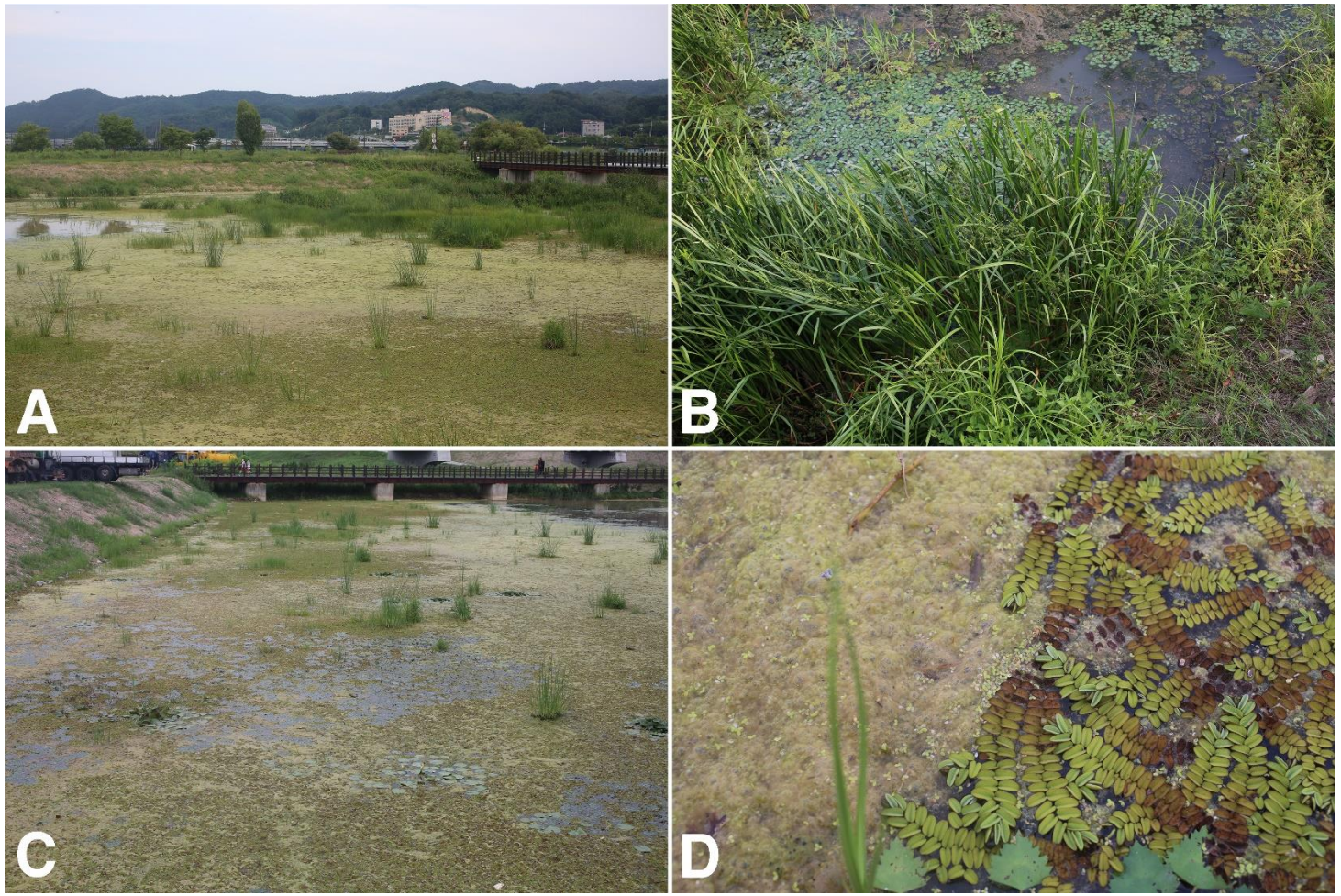


Figure 5.3. The Banbyeoncheon stream in Andong; the native habitat of *Lemna turionifera*: A, The width of the riverbank along the Banbyeoncheon river is ~100 m, and the rate of water flow was not fast.; B, *L. turionifera* was growing amid *Sparganium erectrum* L., *Spirodela polyrhiza* (L.) Schleid., *Trapa sp*.; C, Most of the stream was covered with *Salvania natans* (L.) All., and *Nymphaea* cv. was also present.; D, *L. turionifera* growing with *S. natans* and *Trapa sp*.