Two lineages of Lemna aequinoctialis sensu lato (Araceae, Lemnoideae) based on physiology, morphology, and phylogeny

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Abstract

Lemna aequinoctialis Welw. is a widely spread species that has diverse physiological and molecular properties. In this study, physiological and morphological analyses were conducted by inducing flowering, and molecular analysis was done based on two chloroplast DNA loci (*atpF*–*atpH* intergeneric spacer, *matK*) of *L. aequinoctialis sensu lato* from 70 strains found in 70 localities in Japan, Korea, Thailand, and the US. 752 flowering fronds from 13 strains were observed based on axenic conditions. Two different trends in flower organ development, protogyny and adichogamy, were detected in these strains. Their physiological traits were divided into two groups, showing different morphological features based on frond thickness, root cap, and anther sizes. Molecular analysis showed two lineages corresponding to two physiological groups. These were identified as *L. aequinoctialis sensu* Beppu et al. (1985)d *aoukikusa* Beppu et Murata based on the description of the nomenclature of *L. aoukikusa*. These were concluded as independent taxa and can be treated as different species. Furthermore, the distribution of *L. aoukikusa* is not only limited to Japan.

Introduction

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

Lemna L., its largest genus, is characterized by a solitary root per frond and one pistil with two stamens surrounded by a spathe. Owing to their small size (0.6 – 9 mm), except for *Lemna trisulca* L., and simple and variable organs, it is hard to identify them, limiting their taxonomical boundary and status.

L. aequinoctialis Welw. is one of the most widely spreading species of the genus *Lemna* (Landolt, 1986). This species has been widely studied by various scientists. Taxonomy, genetic diversity, and various responses to environmental control were 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 recent molecular studies reported that *L. aequinoctialis* is a monophyletic species (Bog et al. 2010; Braglia et al. 2021a; Kim et al. 2017; Barbosa Neto et al. 2019). Two flower types, 1) homogamous and self-compatible and 2) protogynous and self-incompatible, were reported in the *L. aequinoctialis* group in Japan (Beppu 1981). Floral mechanisms are well-known as a crucial factor in speciation (Coyne and Orr 2004; Mayr 1947). However, because the flowers of *L. aequinoctialis* are small, rare, and have a short flowering period, the floral mechanisms have been rarely studied and reported (Beppu 1981).

Beppu et al. (1985) noticed differences among *L. aequinoctialis* groups in Japan, and a new Japanese endemic taxon, *L. aoukikusa* Beppu et Murata [we treat *L. aequinoctialis sensu* Beppu et al. (1985)d *aoukikusa* as *L. aequinoctialis* complex, hereafter], was established based on their physiological, morphological, cytological, ecological, and molecular (allozyme) studies (Beppu 1981; Beppu and Takimoto 1981a, b, c; Yukawa and Takimoto 1976). However, the widely overlapping range of their morphological variations did not agree with the taxonomy of other scientists (Halder and Venu 2012; Landolt 1986; Sree et al. 2016). In particular, Landolt (1986) pointed out a limitation of a study by Beppu et al. (1985) that only compared Japanese *Lemna* plants; the greatest physiological features of the new taxa were dismissed as variations of those of *L. aequinoctialis* limited only within Japan due to the thousand years of rice cultivation.

Since Wang et al. (2010) suggested the DNA barcoding for Lemnoideae identification, recent *Lemna* researchers commonly reflect the DNA barcoding region(s) in their studies (Braglia et al. 2021a, b; Chen et al. 2022; Marconi et al. 2019; Suzuki et al. 2014). However, the uncertain taxonomic identification of Japanese *L. aequinoctialis* complex makes it challenging to use DNA sequence data from the International Nucleotide Sequence Database Collaboration. Therefore, by clarifying the physiological and morphological differences between species within the complex, it may be possible to genetically identify them based on DNA barcoding region sequences.

Furthermore, as most *Lemna* species, except *L. aequinoctialis*, were reported to be protogynous and self-incompatible (Les 2020), the presence of two flowering characteristics in one species (*L. aequinoctialis* in Japan; Beppu 1981) suggests two possible hypotheses. First, there may be two distinct species with different physiology, morphology, and genetics within *L. aequinoctialis* (Beppu 1985). Alternatively, the two floral physiological types may be maintained as polymorphic characters within the species, with no dynamic isolation among them.

To elucidate the high diversity of the *L. aequinoctialis* complex, we particularly focused on their floral mechanisms, morphological variations, and relationships with genetic diversity. This study aimed to 1) identify and categorize various properties observed in *L. aequinoctialis sensu lato* and measure the differences between the groups; 2) determine whether genetic features reflect the differences between the groups; and 3) examine the geographic distributional patterns of the groups within the *L. aequinoctialis* complex.

Materials and methods

Sample colletion

From April 3, 2019, to October 4, 2020, *L. aequinoctialis* complex samples were collected from 70 localities of rice fields, lake, and ponds in Japan, Korea, and Thailand (Table 1). The Landolt's strain of *L. aequinoctialis*, strain number (no.) 6746 previously used for studying the 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) was also added. *L. valdiviana* Phil. was also collected in Japan for phylogenetic analysis. Voucher specimens (Table 1) were stored in a herbarium of Niigata University Herbarium (NGU) or Andong National University Herbarium (ANH).

Table 1
Collected *Lemna* strains and their information. The origin of the plants, GenBank accession number, and haplotypes are provided. Except for asterisk marked four sequences from Landolt's strains, all sequences were generated in this study. *: Landolt collection, **: already analyzed in the previous study

No.	Strain	Country	Locality	Latitude & Longitude	Collection date	Accession number		Haplotype		Voucher
						atpF-H	*matK*	*atpF-H*	*matK*	
Lemna 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 & 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)

No.	Strain	Country	Locality	Latitude & Longitude	Collection date	Accession number		Haplotype		Voucher
						atpF-H	*matK*	*atpF-H*	*matK*	
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 & 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)
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 (NGU12753)
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 & 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)

Table 1
continued

No.	Strain	Country	Locality	Latitude & Longitude	Collection date	Accession number		Haplotype		Voucher
						atpF-H	*matK*	*atpF-H*	*matK*	
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)
57	NGY60	Japan	Fukuoka Pref.	33°48'26.1"N, 130°47'57.6"E	Jul. 22 2019	LC752862	LC753040	A13	M6	Lee & 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 & 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 & 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)

No.	Strain	Country	Locality	Latitude & Longitude	Collection date	Accession number		Haplotype		Voucher
						atpF-H	*matK*	*atpF-H*	*matK*	
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 & 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 & 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 & 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 & 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 & Tone (NGU12790)
70	NGY302	Thailand	Thoong Aruan	14°36'N, 102°11'E	unknown	LC752875	LC753053	A11	M7	Lee et al. (NGU14391)
71	6746*	US	California			GU454216**	GU454217**	A6	M4	
L. minuta										
72	9476*	UK	England			MK516250**	LC753054	LA1	LM2	
L. valdiviana										
73	NGY189	Japan	Hyogo Pref.	34°39'01.0"N, 134°59'35.0"E	Sep. 25 2020	LC752876	LC753055	LA1	LM2	Lee et al. (NGU14392)
74	9222*	Bolivia	La Paz			LC752877	LC753056	LA1	LM3	
75	9475*	Brazil	Manaus			MK516244**	LC753057	LA2	LM1	

Establishment of axenic strain

More than 12 fronds from each locality were sterilized with 0.5% of NaClO for 10 min and washed with autoclaved distilled water for 10 min twice in a clean bench. Successfully sterilized single fronds were cultured on semisolid (0.45–0.8% agar) Hutner's medium (Hutner 1953), including 1% sucrose at pH 6.5 in a disposable petri dish (90 mmφ × 20 mm). Cultures were grown at 25°C under 12L:12D photoperiodic light using LED lamps (PF20-S9WT8-D, Nippon medical & Chemical instruments, Osaka, Japan) at 40 ± 10 μmol m^{-2} s^{-1} surface light intensity in the incubator (MIR-253, SANYO, Osaka, Japan). In culture conditions, the strains were mainly propagated clonally through frond division. One representative strain of each locality was established and named with abbreviation "NGY", which was derived from "Niigata University" and one of the author first name "Yuri", and ID numbers (Table 1).

Flower-inducing

To observe the flower development and self-fertilization ability of the *L. aequinoctialis* complex, flowering was induced for 13 strains (NGY14, 33, 59, 122, 123, 128, 140, 142, 178, 180, 196, 302, and 6746) using the modified method of Kaihara et al. (1981), as described below. Fronds from a clonal strain were pre-cultured in a 20^{-1} strength nitrate-free Hutner's medium (with 1% sucrose) with 10 μM benzoic acid in a 100-mL flask for 10 days. To make nitrate-free conditions, 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 an agar-semisolid (0.3%) medium in 90 × 20 mm-sized petri dish to monitor the flower development (Fig. S1). These cultures were incubated at 25°C under 40 ± 3 μmol m^{-2} s^{-1} of continuous light from both LED and fluorescent light (Terukuni Denki, Tokyo, Japan) in an incubator (LH-240N, Nippon medical & Chemical instruments, Osaka, Japan).

For morphometric analysis, including that of flower morphology, flowering was induced with the same conditions in a liquid medium. Flowering was induced in 10 strains (NGY14, 33, 53, 59, 66, 122, 123, 128, 140, 302) in another way, which was based on photoperiodic light treatment

(Landolt 1986; Beppu et al. 1985; Hillman 1975), where 8–12 h of light was offered. In this treatment, the plants were cultured in a liquid Hutner's medium (including 1% sucrose) in a 100-mL flask for over 14 days.

Observation of flower development and self-fertilization ability

Flower monitoring was conducted on 442 flowering fronds (Table S1) from 11 strains every 12 h for seven days under a dissecting microscope (SZ2-ILST, Olympus, Tokyo, Japan). Each frond's flowering moment was recored, and at the final day of observation, the flower organ that matured earlier was determined. New flowers that developed on new fronds during monitoring were also observed. Fronds that bear fruits were counted, and the dropped seed number was recorded to count the frond number that successfully matured on the last day.

Morphometric analysis

The flowers that were induced in two ways did not show significant morphological differences, so all were used for morpholometric analysis. For morphometric measurements, 310 flowering plants from the 13 strains were measured in fresh or immersed (70% ethanol) conditions (Table S2). To observe them in environments similar to natural conditions, all investigated plants were cultured on a liquid medium. We measured previously quoted 15 quantitative characters by Beppu et al. (1985) and Landolt (1986), as well as eight characters that could potentially distinguish *L. aoukikusa* from the *L. aequinoctialis* complex (Table S3). In addition, we focused on two qualitative characters, such as the shapes of root and root tip, because Beppu et al. (1985) suggested that the *L. aequinoctialis* complex can be divided into three types based on these characters. The root characters were observed from the longest one. For anther and filament sizes, the longer stamen was measured. The measured characteristics were statistically tested by Chi-Square test, Mann-Whitney Wilcoxon test, or t-test (Student's test, Welch's test) to determine the significance between the two groups.

To analyze the morphological relationships among the physiological groups, principal component analysis (PCA) was performed. Incompletely measured plants due to early-wilted some organs were excluded. Among the 23 characters, those with a high correlation to each principal factor were selected, and then, those that were highly correlated (value > 0.7) to each other in covariability were removed. Finally, six quantitative characteristics (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).

Phylogenetic analysis

DNA extraction and PCR were conducted based on Lee et al. (2020), except that the PCR primers of *matK* regions were based on Jeanson et al. (2011). *matK* amplification was conducted with 35 cycles of denaturation at 94°C for 1 min 15 s, annealing at 50°C–52°C for 2 min, extension at 72°C for 2 min 15 s, and final extension at 72°C for 5 min.

The products of PCR amplification were purified using exonuclease I (TaKaRa Bio Inc.) and thermosensitive alkaline phosphatase (TSAP; Promega, Madison, Wisconsin, USA). A direct sequencing of purified PCR products was performed using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) using with primers on an automated sequencer (ABI prism 3130 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Purified PCR products were also sent 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). These obtained haplotypes were aligned with the sequences of *L. aequinoctialis* and other close species, including outgroup taxa, analyzed in a 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, Table 1, S4) using ClustalW based on software MEGA-X version 10.0.5 (Kumar et al. 2018) for each locus. The two data matrices of 425 and 581 characters representing 21 and 12 OTUs from five species, respectively, 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. The Tamura's Three-Parameter (Tamura 1992) model was suggested as the best model by the model test in MEGA-X, and it was selected for ML and NJ tree constructions. One of the heuristic searches (branch-swapping algorithm), Subtree-Pruning-Regrafting topology (Nei and Kumar 2000), was used for MP tree construction. All possible subtrees from the 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 two cpDNA regions was performed, but only two species, *L. minuta* Kunth and *L. valdiviana* Phil., were used as outgroup.

Results

Diversity in sexual reproduction

Among the 13 strains of *the L. aequinoctialis* complex, flowering was successfully induced in 11 strains (except NGY140 and 178) by our benzoic acid-adding treatment. The *L. aequinoctialis* complex (Fig. 1a) had two typical phenomena in their sexual organ development. The first

was protogyny, where the pistil first reached out and matured (Fig. 1b). Even though the pistil completely matured and a 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 going outside the frond. However, the pistil usually started withering when the stamen started to be outside the frond.

The other was homogamy, where the pistil and stamen(s) matured the same time. In almost all homogamous flowers, one stamen started to emerge first, and then the pistil followed. They grew almost together, and the anther opened. We could often observe the healthy stigma under the opened anther (Fig. 1c). When the first grown stamen fully ripened and the anther was opened, the second stamen emerged. Moreover, some flowers self-pollinated. The floral organs of both protogyny and homogamy types wilted in 24 h.

We observed 9–82 flowers from each strain (Fig. 2, Table S1). Among 39 flowers from NGY14, 37 flowers had matured pistil and stamens simultaneously, while the pistil in two flowers matured earlier than the stamens. In NGY33, 25 homogamous flowers were observed. In NGY59, 37 out of 38 flowers had matured pistil and stamens simultaneously, with only one protogynous flower. NGY122 bloomed the largest number of flowers, with 79 flowers that had pistils that matured earlier than the stamens, except for three homogamous flowers. In NGY123, all 21 flowers were protogynous. NGY128 had the smallest flower number; eight flowers were protogynous, and one flower was homogamous. In NGY142, 31 flowers developed pistil and anther together, but two flowers developed pistil earlier. In NGY180, 196, and 302, the gynoecium of 61, 27, and 23 flowers matured earlier than the androecium, respectively; on the other hand, the androecium and gynoecium of one, eight, and two flowers matured together, respectively. At last, all 73 flowers of *L. aequinoctialis* no. 6746 were homogamous.

Based on the number of fronds with specific physiological traits from each strain, the strains could be classified into two groups: A (NGY 122, 123, 128, 180, 196, 302) and B (NGY 14, 33, 59, 142, Landolt's *L. aequinoctialis* no. 6746), with over 70% of fronds with pistils that matured earlier than the stamens or with pistil and stamens that matured at the same time, respectively. The rate of protogyny in group A was 74.19% –100%, while the rate of homogamy in group B was 93.94%–100% (Fig. 2, Table S1).

Confirmation of self-fertility

During seven days of flower monitoring, 234 and 208 flowers were observed from groups A and B, respectively. Some self-pollinated flowers successfully fruited. The top of ripe fruits spontaneously bursted, and one seed dropped from one fruit. Three fronds bearing fruits were found in strain NGY122 (group A). The fronds had an enlarged ovary, and they seemed like normal fruits. However, they withered without bearing a seed set. Eventually, we could not find any seed from group A despite the abundant flowers (Table 2).

Table 2
Number of flowering and seed-bearing fronds. All strains were fixed on semisolid medium and self-pollinated spontaneously. Seeds were completely matured and naturally dropped from the fruit. The seed set ratio was calculated based on flower and seed number. The groups were divided based on physiological traits.

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

On the other hand, group B fruited with mature seeds. The rate was 19.18%–100%, and 63.34% on average. Landolt's no. 6746 had the second highest flower number in 11 strains, but it had the lowest fruit and seed number. The fruiting rate was 19.18%. Except for the strain, we could get seeds from over 50% of fronds among flowered fronds (Table 2).

Morphometric analysis

The quantitative, qualitative, and morphological characteristics of 310 flowering fronds from 13 strains were determined (Table S2). The flowers were induced by 8 h of light in NGY14, 33, 53, 59, 66, and 140 or by benzoic acid in NGY 14, 33, 59, 122, 123, 128, 142, 180, 196, and Landolt's *L. aequinoctialis* no. 6746. The characters were compared between the two groups, which were distinguished by physiological traits. In 23 quantitative morphological characters, the ranges of most characters overlapped and no differences between the two groups were found; however, in six characters (frond thickness, root cap diameter, style length and diameter, ovary length, anther length, and diameter), the two groups differed (Fig. 3a, b, d, e, g, h, Table S3). The frond thickness dimensions in groups A and B were 0.63 ± 0.09 mm and 0.57 ± 0.12 mm in average, respectively ($P < 0.0001$, Welch's test). The average root cap diameters were 0.19 ± 0.02 mm and 0.17 ± 0.03 mm, respectively ($P = 0.0002$, Welch's t-test). The mean anther lengths were 0.18 ± 0.03 mm and 0.14 ± 0.02 mm, respectively ($P < 0.0001$, Welch's t-test), and the diameters were0.15 ± 0.02 mm and 0.12 ± 0.02 mm, respectively ($P < 0.0001$, Welch's t-test).

Moreover, 53.27% of fronds in group A had straight roots, while 44.86% had wavy roots. Most plants in group B had straight roots (91.30%). Only two and one fronds had spiral roots in groups A and B, respectively (Fig. 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%) in group A, followed by intermediate and obtuse root tip shape (35.92, 7.77%). On the other hand, almost all fronds in group B had an acute root tip (98.48%), and only one frond had an intermediate root tip (Fig. 4b). Statistically significant evidence also supported the difference between the two groups ($P < 0.0001$, Pearson test).

In the PCA, the two groups could be distinguished along PC axis 1 (Fig. 5a). The ranges of PC values along axis 1 were − 0.567–3.676 for group A and − 4.147–1.834 for group B. When the PC scores were averaged for each strain, the trend between the two groups was clearly different (Fig. 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 the 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 the total variance, was contributed by spathe length, spathe diameter, stigma width, ovary length, filament diameter, and anther length (Table S5).

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 the US (California). In total, there were two insertions and/or deletions and 1–27 substitutions among 14 haplotypes (Table S6). Among them, haplotype A13 from Japan and Korea accounted for 47.1% (n = 72). Haplotype A3 from two lakes in China occupied the next (33.3%; n = 51). The phylogenetic trees (ML, MP, and NJ) showed two clades in the *L. aequinoctialis* complex (Fig. 6a). Haplotypes A1–5, 7–9, 11, and 12 were included in clade α, while A6, 10, 13, and 14 were included in clade β with *L. perpusilla* Torrey. The haplotype A10 was treated same with one of *L. perpusilla* 8539 in our data set. The two clades were supported by bootstrap values (BV) of 43–54%.

In the *matK* region, seven haplotypes were detected (626–853 bp) from 128 *L. aequinoctialis* complex samples from Brazil, China, Japan, Korea, and the US (California). These haplotypes were identified by 1–17 substitution sites (Table S7). Haplotype M1 (48.4%; n = 62) was collected from two lakes in China and Japan, and it dominated all haplotypes. Besides, haplotype M6 (46.1%; n = 59), which got the second dominance, was only found in Japan. The phylogenetic tree also showed two clades in the *matK* region: M1–3 and 7 from Brazil, China, Japan, Thailand, and US (California) were in clade γ, while M4–6 from Japan, Korea, US (California, Texas), and *L. perpusilla* were in clade δ (Fig. 6b). The BVs of the two clades were 99%.

We could detect 10 haplotypes in the two loci, *atpF–atpH* IGS and *matK*, concatenated sequences (1071 bp) from 125 plants of the *L. aequinoctialis* complex from China, Japan, Korea, Thailand, and the US (Table 1, S5). The phylogenic trees showed the two monophyletic lineages in 10 haplotypes (Table 3, Fig. S2). The *atpF–atpH* IGS (A) and *matK* (M) haplotype combination; types 1–7 were included in clade 1, and types 8–10 were included in clade 2. There were no major differences in topology with the former two trees based on each locus (Fig. 6a, b), but two clades in the *L. aequinoctialis* complex were more highly supported (BV > 89% and 79%).

Table 3
The 10 haplotypes concatenated two chloroplast DNA regions of Lemna aequinoctialis complex collected from China, Japan, Korea, Thailand, and US. Each letter in square brackets indicates clade in phylogenetic analysis. "n" indicates the number of sequences. 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. The groups were divided based on physiological and morphological traits.

No.	Haplotype			n	Group
	atpF–atpH	*matK*	concatenate		
1	A1 [α]	M1 [γ]	Type 1 [1]	8	A
2	A2 [α]	M1 [γ]	Type 2 [1]	2	A
3	A3 [α]	M1 [γ]	Type 3 [1]	50	–
4	A3 [α]	M2 [γ]	Type 4 [1]	1	–
5	A4 [α]	M1 [γ]	Type 5 [1]	1	–
6	A5 [α]	M1 [γ]	Type 6 [1]	1	–
7	A11 [α]	M7 [γ]	Type 7 [1]	1	A
8	A6 [β]	M4 [δ]	Type 8 [2]	1	B
9	A10 [β]	M4 [δ]	Type 9 [2]	1	–
10	A13 [β]	M6 [δ]	Type 10 [2]	59	B

Haplotype distribution

A distribution map was made based on the haplotypes of Japan and Korea's *L. aequinoctialis* complex (Fig. 7). In Japan, three haplotypes (A1, 2, and 13) were distributed. Among them, A13 was widely distributed in Japan; on the other hand, the distributions of A1 and A2 were limited to the southern part of Japan. The haplotypes in Korea were more varied than in Japan; there were not only A1 and A13, which were distributed to Japan, but A8, A9, and A14 also exist in Korea.

Discussion

Physiological difference

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

We could also find both traits not only in Japan but also in Korea, Thailand, and the US. During our monitoring of flower organs' maturation timing, the pistil and stamen(s) of some fronds in both groups matured at the same time (Fig. 1c, 2). In this situation, without continuous (every 12 h) monitoring, it was difficult to judge which organ grew in advance. These characteristics were also found in Khurana et al. (2011). However, there was no mention about the flower development difference between the two strains of *L. aequinoctialis*. Landolt (1986) mentioned that he never saw protogyny in *L. aequinoctialis*. We think that it is because of the difficulty of inducing flowers in *L. aequinoctialis sensu* Beppu et al. (1985). Beppu et al. (1985) mentioned that *L. aequinoctialis* does not flower without adding salicylic acid. At that time, they cultivated them in a greenhouse to get flowers. Similarly, we could not induce flowering in group A using photoperiodic control, but it was induced using benzoic acid. Moreover, we could sometimes find flowers under natural conditions in summer (locality of NGY180, 184, 196, and 199 in clade 1; Fig. S3a) like 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. S3).

Under benzoic acid-added conditions, it was sometimes difficult to get seeds from some strains. At the beginning of the observation stage, the low fruiting rate of group A seemed to be due to self-sterility resulting from differences in flower parts' maturation timing. Hence, we tried to pollinate artificially in each strain, but we also could not obtain any mature seeds (Lee, unpublished data). In strain NGY122, although we observed three fruits, they did not successfully mature (Table 2). These results indicate that the plants of the physiological group A have self-incompatibility. Landolt (1957) mentioned the possibility of self-fertile and self-sterile in *L. perpusilla*, which is closely related to *L.*

aequinoctialis. He discovered a matured fruit set from strain no. 6746, which belonged to physiological group B and phylogenetical clade 2 in this study (Fig. 1, 2, 6, S2, and Table 2) 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* may also 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 rates under four conditions, he concluded that S type is self-incompatible. Beppu (1981)'s S type may be physiological group A. More detailed pollination studies are needed for self-incompatibility of the *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. We could confirm that NGY122 and 123 (group A) were living as frond in January in Okinawa (Table 1).

Here, we detected the various physiological mechanisms in the *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 angiosperms after the Cretaceous Period (Whitehouse 1950). It is known to be one of the important mechanisms in evolution and specification (Ferrer and Good 2012). The different overwintering types between the two groups might be influenced by their different pollination mechanisms, and it also affected their geographical distribution.

The Morphology of L. aequinoctialis complex

Our research indicated that the physiologically distinct two groups A and B had different trends in morphological characteristics. 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 that of *L. aoukikusa*. Our research also shows the different trends in these characteristics between the two physiological groups (Fig. 5). However, while they argued that 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) was not much different with *L. aoukikusa* (0.64–1.07 and 0.64–1.21 mm), the size of former character was the same in the two physiological groups (Fig. 3d, Table S3), the latter character showed longer size in group A (0.60 ± 0.13 mm) than group B (0.52 ± 0.14 mm) (Table S3).

The nomenclators of *L. aoukikusa* treated the root and root tip shapes as diagnoses (Beppu et al. 1985). Although we could not clearly divide two physiological groups based on these characteristics, groups A and B showed different trends (Fig. 4). And we also found different trends in the root cap diameter between the groups (Fig. 3b). Root morphology is probable that taxonomically candidate character for the identification of these groups. Landolt (1986, 1998) argued that the root cap of the subfamily Lemnoidae never regenerates, unlike in land plants, and at the same time, 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 mixtured 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). We could not compare the fruit and seed characteristics 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 a significant difference between the two groups in the *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 the world level of *L. aequinoctialis*, the discovery of differences in not only vegetative but also sexual morphological characteristics between the two groups cultured under the same condition suggests that the independence of group B as a species-level from group A.

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, we added four close species: *L. minuta*, *L. perpusilla*, *L. tenera*, and *L. valdiviana* as outgroup for phylogenetic analysis. In both the phylogenetic trees based on two loci, *L. perpusilla*, which belongs to sect. *Alatae* were grouped with the *L. aequinoctialis* complex. It shows they are closely related, and it can support the validity of their taxonomical position in same section. Meanwhile, there were sometimes discordance in sequences from the same sample (*L. perpusilla* 8539 and *L. tenera* 9020, 9024) by different researchers (Bog et al. unpublished; Borisjuk et al. 2015). It is possible that some sequences of *L. perpusilla* and *L. tenera* are wrong, probably due to errors in the experimental process. Since we could not determine which sequence was incorrect, we included both in the phylogenetic tree.

All *L. perpusilla* belonged to clade β with group B. The sequence of *L. perpusilla* 8539 (MG775397) and *L. aequinoctialis* 7126 (GU454217) had one substitution in *atpF* – *atpH* IGS but the valid substitution was cut, they were treated identical in this phylogenetic analysis. The strains *L. aequinoctialis* 6612, 6746, 7126 (haplotype A3, 6, 10, respectively) and *L. perpusilla* 8539, 8612 have been identified based on morphology (and/or physiology) in past and the allozyme-based molecule data supported their classification (Crawford et al. 2001). Thus, the independence of *L. perpusilla* from *L. aequinoctialis* (including *L. aoukikusa*) may be not wrong. Kandeler and Hügel (1974) argued that whether seed drops out from ripe fruit is an important character of *L. aequinoctialis* and *L. perpusilla*. In this study, we observed the seed naturally dropped down from fruit in group B. However, we could not confirm the seed-drop-characteristic of *L. aequinoctialis* 7126 strain which belonges to clade β. Therefore, inner relationship of clade β needs to be carefully considered for taxonomic treatment in the future.

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 (Yamaga et al. 2010) to their study. They offered BLASTN result of the 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 the *atpF* – *atpH* region and concluded that its noncoding DNA sequence is identical to which of *L. aequinoctialis*. Based on the result, they reidentified the sample as *L. aequinoctialis*. However, unfortunately, we could not judge the Hokkaido's *L. aoukikusa* in detail only by the mismatching number. Considering our result that there were three to eight substitutions (when insert/deletions were deleted) between clades α and β (Table S6), we can not exclude the possibility that the Hokkaido's plant is *L. aoukikusa*. As such, it may cause a problem to simply identify species only by the substitution number or matching rate in DNA sequence.

The haplotype distribution (Fig. 7) indicates different distribution patterns in the two clades. Compared with the distribution map in Beppu et al. (1985), clade α's distribution corresponded to of *L. aequinoctialis sensu* Beppu et al. (1985), clade β's distribution agreed with of *L. aoukikusa*. This supports the possibility that the two clades represent the two *Lemna* species.

Conclusion

This study aimed to elucidate the high diversity of the *L. aequinoctialis* complex. Our results successfully revealed the following. 1) The floral characteristics (flower organs' mature timing, self-compatibility, or self-incompatibility) of the *L. aequinoctialis* complex were differentiated. Moreover, root cap diameter was newly discovered as a simple key character for taxon identification. 2) Two groups in the *L. aequinoctialis* complex, distinguished by physiological and morphological traits, corresponded to two genetically divided clades in cpDNA. Future studies using nuclear-based molecular analysis are needed to reveal more details in their evolutionary relationship. 3) The different geographical distributions of the two groups may serve as a representative character. However, further studies are required to understand the properties that influence their distribution. 4) Our findings indicate that *L. aequinoctialis* and *L. aoukikusa* are different species and we recommend that the latter species be recognized.

Declarations

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Competing interests

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Figures

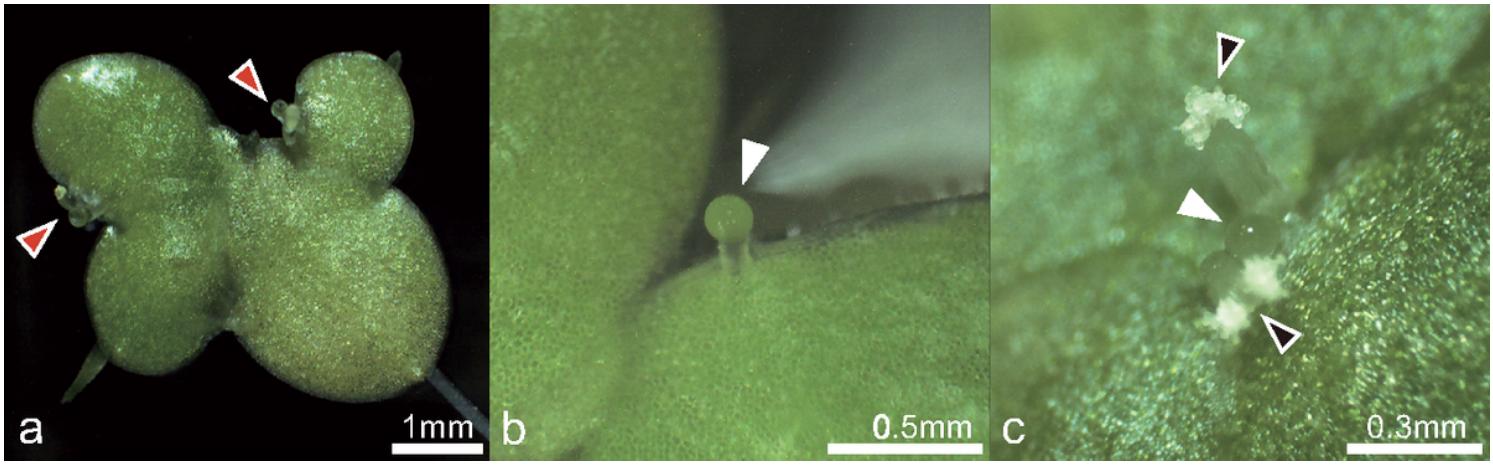

Figure 1

Flowers of *Lemna aequinoctialis* complex. **a**A whole plant of flowering *L. aequinoctialis* complex (NGY13); 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 matured completely and opened; the droplet is on the stigma (NGY33).

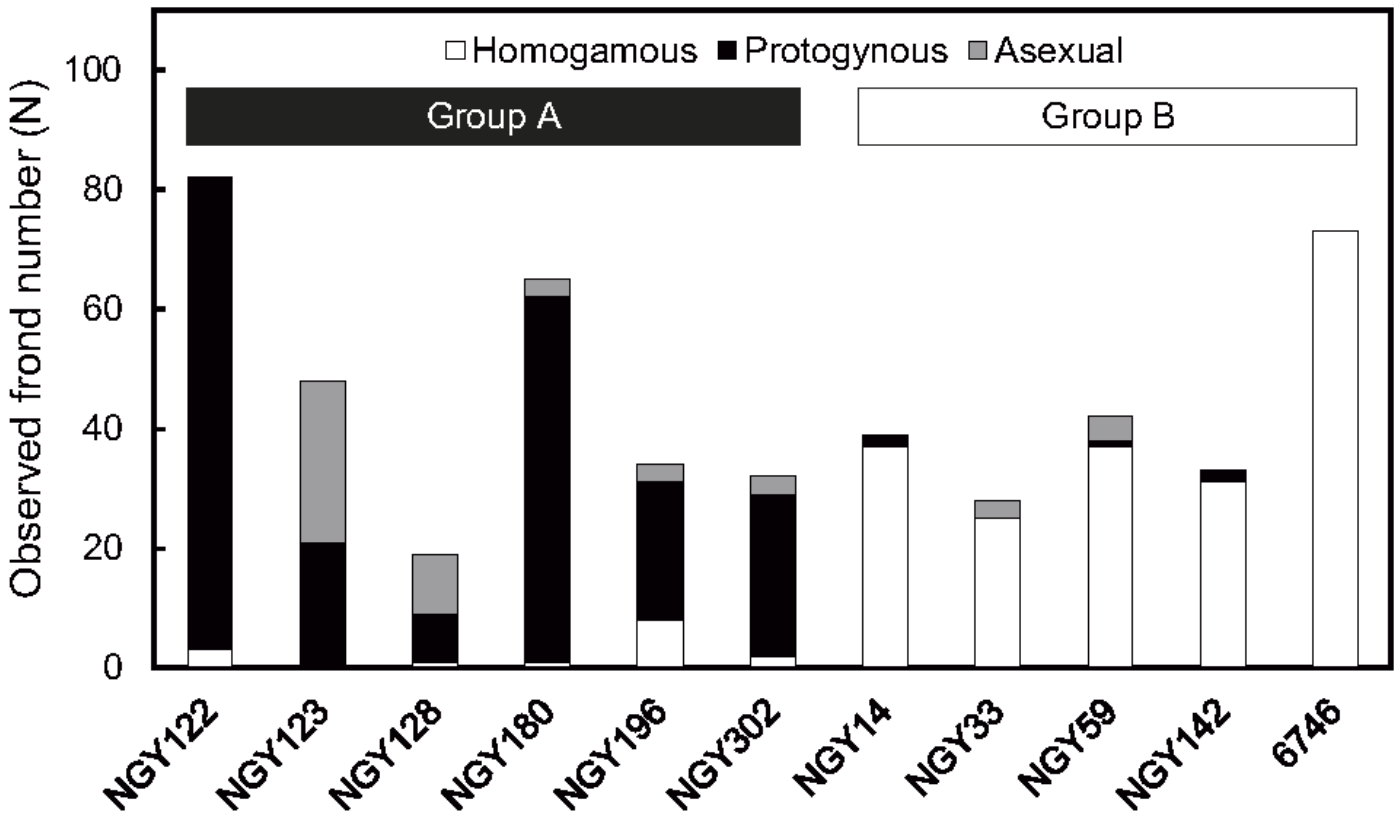

Figure 2

Flower organ's mature timing of each strain. The flowers were observed every 12 h for 7 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 simultaneously. The gray color indicates not-bloomed frond number.

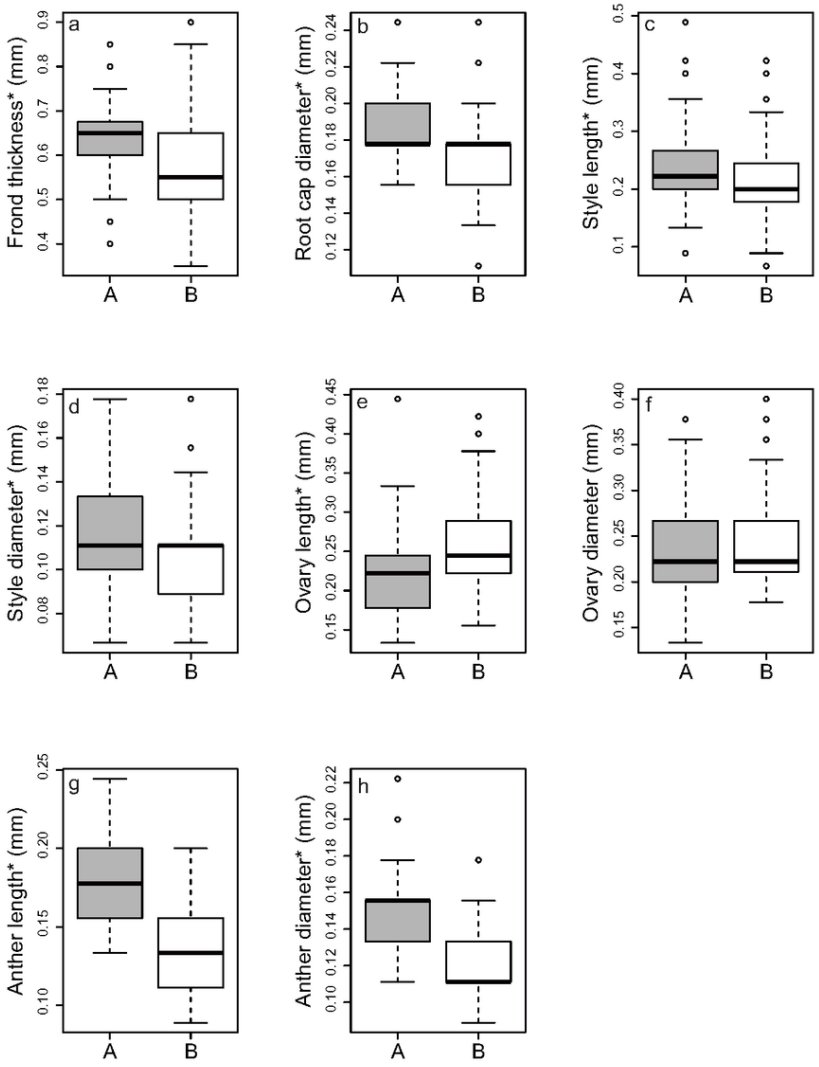

Figure 3

Eight morphological characteristics of physiologically divided two groups in *Lemna 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 (*) character indicates statistical difference in two groups. For each box plot, the top bar indicates the maximum value, the top end of the box indicates the third quartile, the middle bar indicates the median value, and the circles indicate possible outliers.

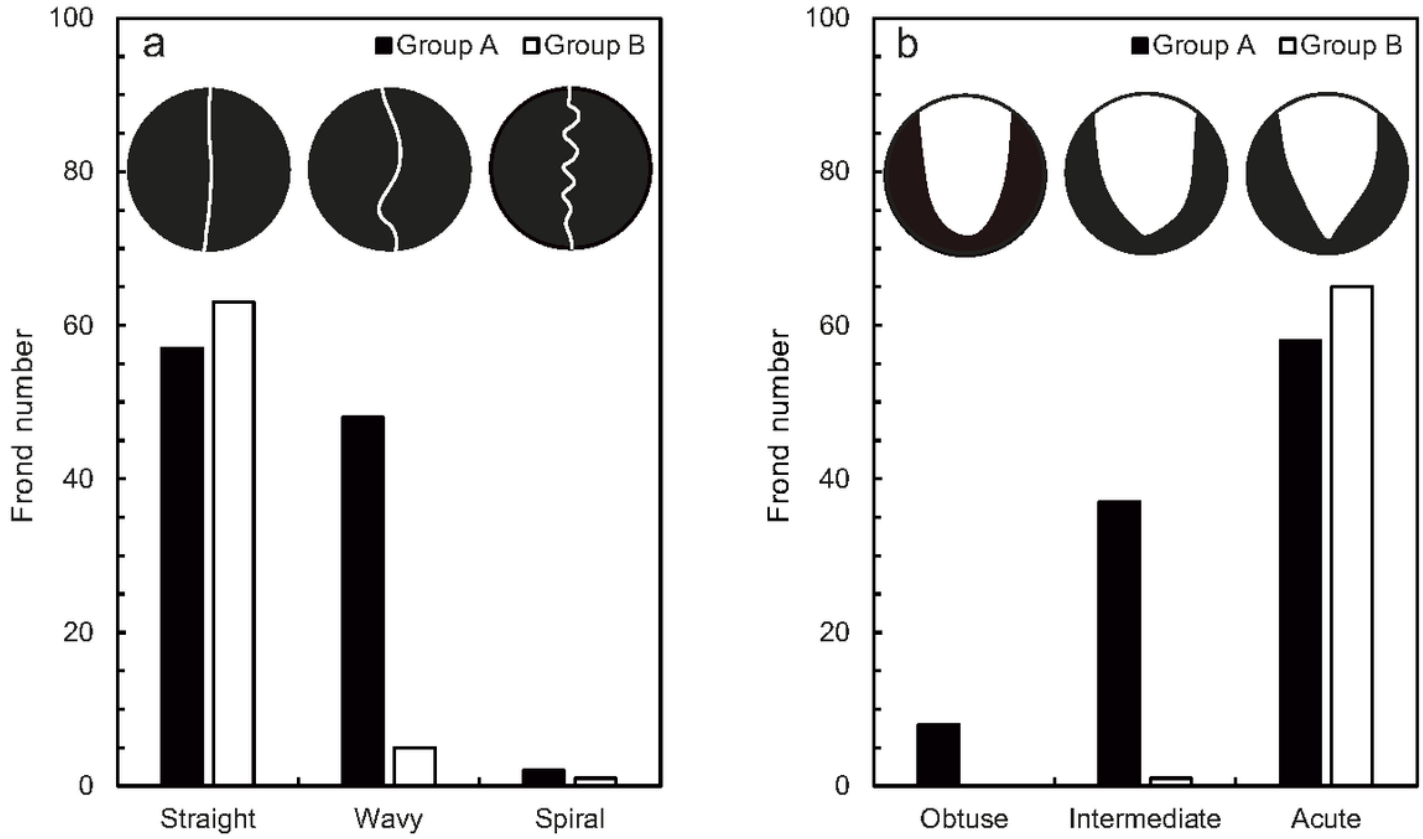

Figure 4

Root morphology of *Lemna aequinoctialis*complex. **a** The number of fronds which has a straight, wavy, or spiral root shape on two groups. **b** The number of fronds which has an obtuse, intermediate, or acute root tip on two groups.

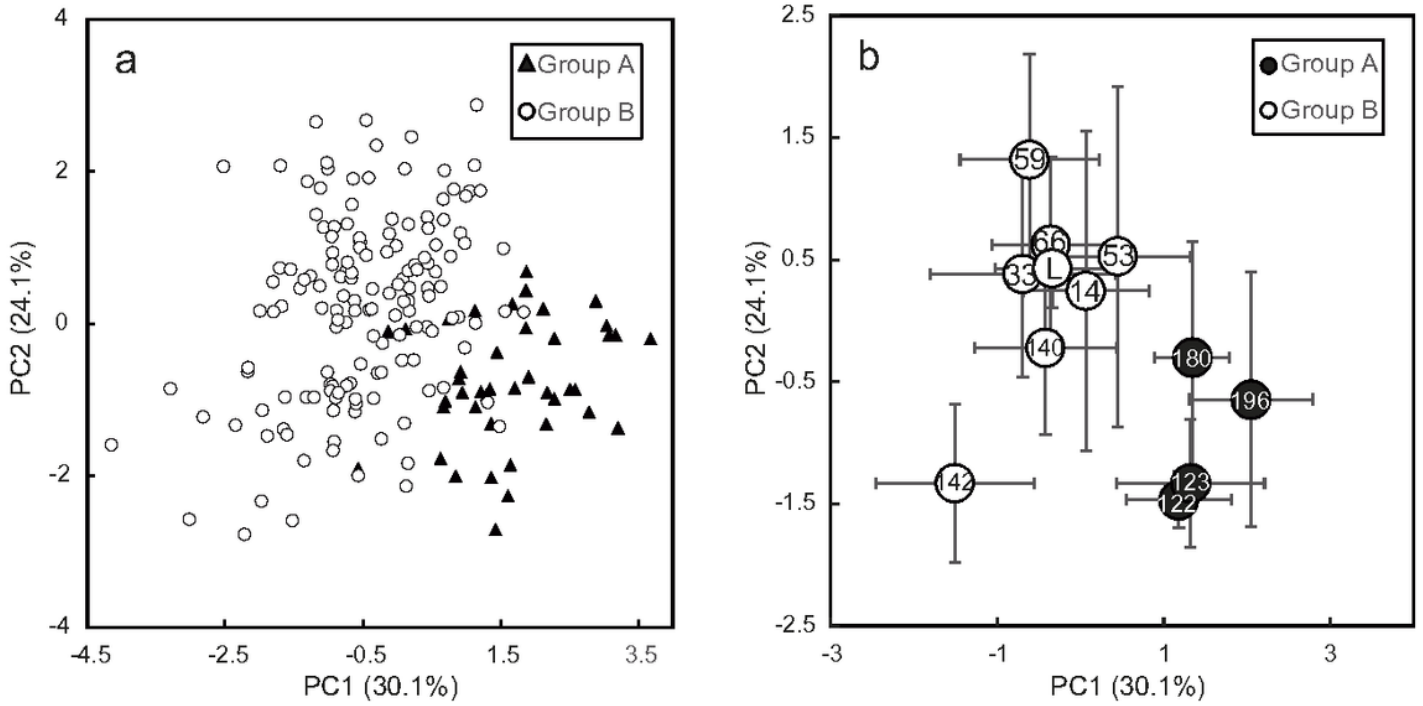

Figure 5

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

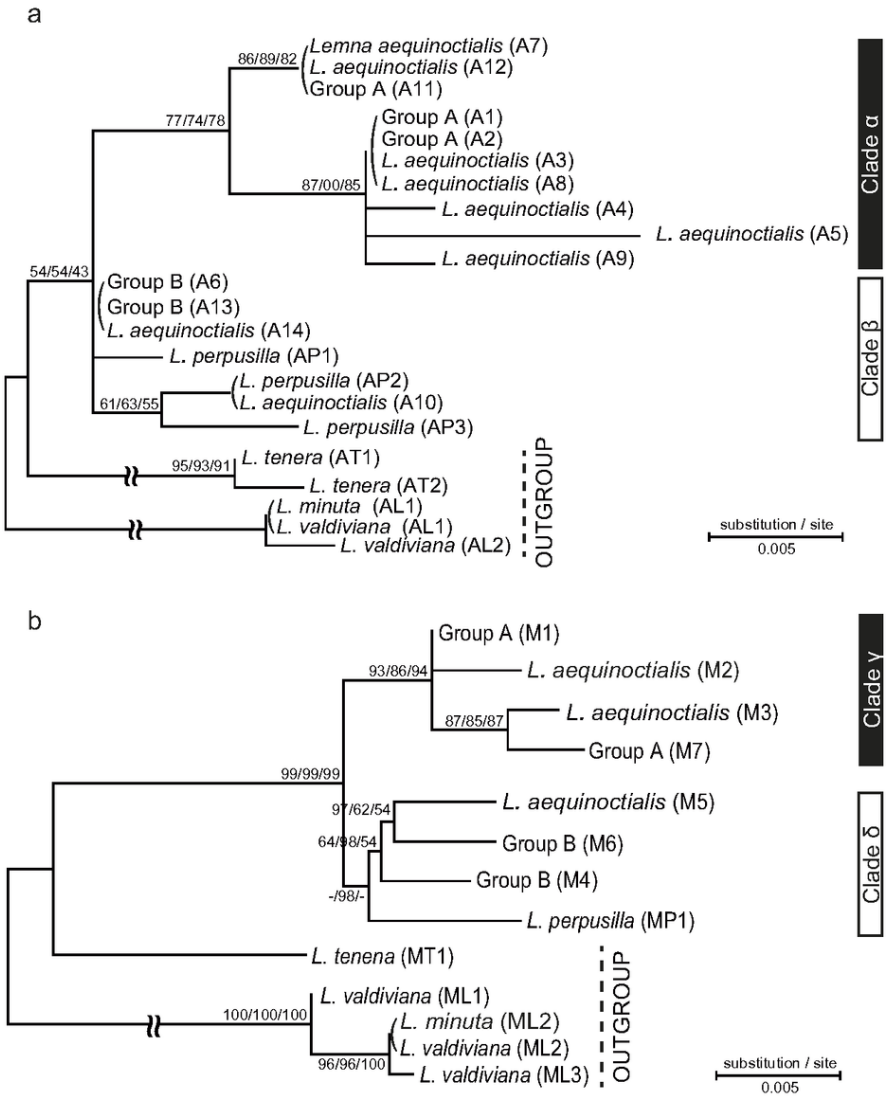

Figure 6

Neighbor-joining trees of **a** *atpF–atpH* and **b***matK* based on 425 and 581 aligned characters of cpDNA of 21 and 12 haplotypes of *Lemna aequinoctialis* complex (Tables S6, S7) and outgroup. Bootstrap values (ML/MP/NJ) are indicated on branches.

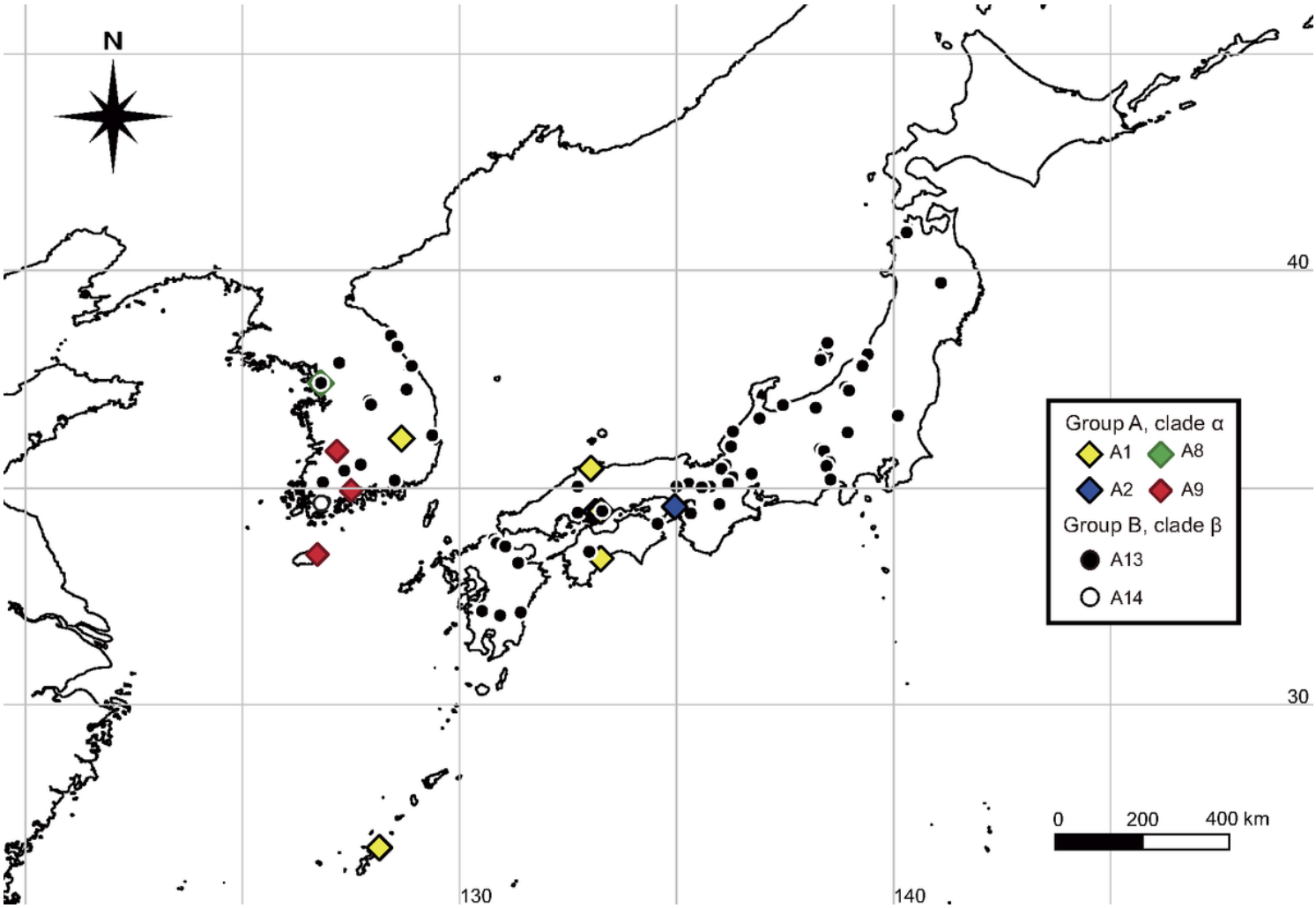

Figure 7

Geographic distribution of the six cpDNA haplotypes in Japan and Korea. The samples investigated only in *atpF–atpH* are also shown (A1, A2, A8, A9, A13, and A14). Most cpDNA information of Korean *Lemna aequinoctialis* complex was collected from Kim et al. (2017).

Supplementary Files

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