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Triploidy is prominent in the duckweed *Lemna minor* complex

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Todd P. Michael[1,2,3] ✉, Phuong T. N. Hoang[4,5], Bradley W. Abramson[1,14], Allen Mamerto[1], Buntora Pasaribu[6], Jörg Fuchs[4], Evan Ernst[7], Nicholas Allsing[1], Luca Braglia[8], Semar Petrus[1,15], Veit Schubert[4], Nolan Hartwick[1], Megan Wang[6], Yi-Feng Chen[6,9], Mariele Lensink[1,13], Tiffany Duong[1], Kelly Colt[1], Manuela Bog[10], K. Sowjanya Sree[11], Laura Morello[8], Klaus-J. Appenroth[12], Ingo Schubert[4], Robert A. Martienssen[7] ✉ & Eric Lam[6,9] ✉

Duckweeds (Lemnaceae Martinov) are aquatic monocotyledonous plants known to be the smallest and fastest-growing flowering plants on Earth. Many species are morphologically indistinguishable due to their reduced structures, yet molecular evidence suggests that visually similar clones could be distinct species or hybrids. Using a comprehensive suite of genomic, molecular and cytogenetic methods, we analyzed 58 presumed *Lemna minor* clones to resolve their relationships. Our study revealed extensive genome plasticity within the "*Lemna minor* complex," identifying diploid and triploid *L. minor* clones, as well as allodiploid and allotriploid interspecific hybrids, in addition to a novel African-clade *L. minor* lineage. Triploidy in the *L. minor* complex is prevalent in nature, occurring in 33% of the clones previously identified as *L. minor* in our collection, and is associated with enhanced growth metrics under optimal conditions. For asexually propagative species such as duckweeds, where the triploid block is not a deterrent, triploid progenies may become the majority as has been observed in numerous wild *Lemna* populations. Our study shows that polyploidy and hybridization in these primarily asexually propagating plants could play important roles in their population dynamics and evolution under changing environments.

Duckweeds (family Lemnaceae) are rapidly gaining recognition as versatile organisms with broad applications, owing to their simple body plan, rapid growth, and adaptability to various aquatic environments[1]. They are emerging model plants whose small size and fast life cycles facilitate genetic and physiological research, providing insights into fundamental biological processes[2,3]. Their high protein content has also spurred interest in duckweed as a sustainable feed supplement and a potential food source, including for bioregenerative life-support systems in space[4–6]. Together with other attributes amenable for sustainable food and fuel production[7–9], duckweed is a promising resource to address critical challenges in food security, environmental sustainability, and innovative plant research on Earth and beyond.

The Duckweed family comprises 35 species and three nothospecies across the five genera *Spirodela*, *Landoltia*, *Lemna*, *Wolffiella*, and *Wolffia*, which vary in size from about 15 mm down to less than 1 mm[2]. *Spirodela*, the earliest-diverged duckweed genus, has the largest fronds and produces 10–15 roots. In contrast, *Landoltia* and the single-rooted *Lemna* species have smaller fronds and fewer roots. *Wolffia* and *Wolffiella* represent the smallest and most recently diverging genera which lack roots altogether, while some *Wolffia* species can double their biomass in less than 24 h[10]. Although duckweeds primarily reproduce asexually by budding new fronds from progenitor fronds, most species can also reproduce sexually via flowering and seed formation under suitable conditions[11,12]. More recently, three nothospecies involving hybrids between species in the *Lemna* genus have been recognized, which demonstrated that interspecific hybrids could be prevalent in evolution of this family[13–15].

Since duckweed predominantly grows by clonal propagation, and flowering as well as fertile seeds are rare for most species, they must be maintained as a living collection to facilitate researchers working on the same genetic backgrounds. Therefore, Prof. Elias Landolt established and maintained the Landolt Duckweed Collection, which was started more than 70 years ago. Many of the accessions from this collection are now housed at the Rutgers Duckweed Stock Cooperative (RDSC; http://www.ruduckweed.org/), and the National Research Council, CNR, Milan, Italy (https://biomemory.cnr.it/collections/CNR-IBBA-MIDW), together representing more than 1000 clones across all five genera and their species collected worldwide by researchers from 1953 to the present[11]. In our attempts to characterize this collection from the genome perspective, genome sizes were

A full list of affiliations appears at the end of the paper. ✉e-mail: toddpmichael@gmail.com; tmichael@salk.edu; martiens@cshl.edu; eric.lam@rutgers.edu

estimated from selected clones revealing an order of magnitude difference across the family from 160 megabases (Mb) in *Spirodela polyrhiza* clones to 2203 Mb per haploid genome (1 C) in some *Wolffia arrhiza* clones[2,16–19]. Considerable intraspecific genome size variation was also noted for some species like *L. minor*, which ranged from 356-604 Mb, and yet the differences did not correlate with increased ribosomal DNA or other features tested[16]. The problem with *L. minor* is that it is hard to distinguish morphologically between different accessions and closely related *Lemna* species[20,21] (Fig. S1). Recent molecular and genomic evidence suggests that clones labeled as *L. minor* in the RDSC may represent different species or previously cryptic hybrids such as *L. minor* × *L. turionifera* (*L. ×japonica*) and *L. minor* × *L. gibba* (*L. ×mediterranea*)[13,14,22–25]. Therefore, the exact species identity for most of the *L. minor* clones in the collection remains ambiguous.

In this work, we systematically evaluated all 58 clones originally designated as *L. minor* housed at the RDSC to determine their species identity and genomic traits. Our approach integrated whole-genome sequencing (WGS), flow-cytometric genome size measurements, molecular markers, chromosome counting, and genomic in situ hybridization (GISH). We also leveraged our previous chromosome-scale genomes of *L. minor*, *L. gibba*, *L. turionifera*, and *L. ×japonica* along with short read (SR) Illumina assemblies to construct a super-pangenome and examine genome dynamics within the "*Lemna minor* complex". This encompasses a clade of morphologically similar and genetically interconnected duckweed species, whose close evolutionary relationships, frequent hybrid formation, and subtle morphological differences make species delimitation challenging[10,23,25–27]. We clarified genome composition, ploidy levels, relatedness, and evolutionary origins of the analyzed clones, thereby explaining the observed variability in genome size and chromosome number. This new clarity in genome composition and identity provides the foundation to begin a systematic comparison between ploidy and growth-related traits using the newly defined groupings among these and future *Lemna* isolates.

Results

Resequencing *L. minor* from the RDSC duckweed collection

We analyzed all 58 clones designated as *L. minor* from the Rutgers Duckweed Stock Cooperative (RDSC) (http://www.ruduckweed.org/) by Illumina SR resequencing to investigate the genomic variation and potential misidentifications. As noted above, recent studies have reported that some presumed *L. minor* clones in the RDSC are actually hybrids, specifically *L. minor* × *L. turionifera* (*L. ×japonica*) and *L. minor* × *L. gibba* (*L. ×mediterranea*)[13,14,22,25]. Assuming a 1 C genome size of ~400 Mb, we targeted a median sequencing depth of 60x coverage per haploid genome (Supplementary Data S1, S2). We mapped the reads to a concatenated reference genome of *L. minor* (Lmin9252) and *L. turionifera* (Ltur9434) to detect hybrid presence as well as their genome composition[25]. The overall mapping rate was 98% for most of the clones except four (9425a [67%], 7264 [29%], 5635 [30%], and 8292 [11%]) (Table 1; Supplementary Data S1). Recent barcoding studies using TBP (Tubulin intron-Based Polymorphism) identified clone 9425a as *L. ×mediterranea*, a triploid hybrid between *L. minor* and *L. gibba*, containing one *L. minor* genome and two *L. gibba* genomes (MGG)[14,28]. We remapped clone 9425a to a concatenated version of the *L. minor* (Lmin9252) and *L. gibba* (Lgib7742a) genomes, which increased mapping from 67% to 98% confirming that this clone was *L. ×mediterranea*.

The percent of the genome covered revealed three distinct groups: 1) 53.4% (31/58) were *L. minor* clones, mapping with high coverage (~92%) to the *L. minor* genome and low mapping to *L. turionifera* (6 to 42%); 2) 41.4% (24/58) were *L. ×japonica* (*L. minor* × *L. turionifera*) hybrids, mapping with high coverage (95%) to both *L. minor* and *L. turionifera*; and 3) two clones (7264, 5635) deviated from this pattern, mapping with lower coverage to both genomes consistent with their low overall mapping rates (Table 1; Supplementary Data S1, S2). Clone 8292 with the overall mapping rate of 11%, covered the *L. minor* and *L. turionifera* genome at 91% and 6% respectively, suggesting that it was in fact *L. minor*; however, the low overall mapping rate was due to bacterial contamination (~88% *Stenotrophomonas*). Since clones 7264 and 5635 had lower overall mapping rates and low (~7%) coverage of the *L. minor* and *L. turionifera* genomes, we mapped it against other published duckweed genomes and found that both clones mapped at a high overall rate and coverage to *Landoltia punctata*[23] (Table 1, Supplementary Data S1, S2). Our results confirm that three presumed *L. minor* clones in the RDSC have been misidentified, revealing a new hybrid of *L. ×mediterranea* (9425a) and two from the genus *Landoltia* (7264, 5635).

Next, we looked at coverage depth across the clones to estimate ploidy since we have shown that *L. ×japonica* clones consist of allodiploid (a specific type of hybrid that has one haploid genome from two distinct species, which has also been referred to as homoploids) and triploid hybrids[25,29], which could explain the difference in genome size estimates. All the predicted *L. minor* clones mapped with *a* ~ 53x and ~2x coverage depth to the *L. minor* and *L. turionifera* genomes respectively, while in contrast, the *L. ×japonica* clones mapped at 33x and 22x coverage depth, respectively. Therefore, all clones were sequenced to depths approaching the intended 60x coverage depth, except clone 8292 that has a significant number of bacterial reads. The *L. ×japonica* clones further separated based on the ratio of *L. minor* (M) to *L. turionifera* (T) genome coverage depth into ratio of ~1, ~2, and ~0.5, which suggested that we have 7 MT allodiploids, 16 MMT triploids and one MTT triploid, respectively[25]. Therefore, the presumed *L. minor* clones were 53.4% *L. minor*, 41.4% *L. ×japonica*, 3.4% *Landoltia punctata*, and 1.7% *L. ×mediterranea*.

K-mer genome size and heterozygosity estimate

While we successfully clarified the species identity of the 58 presumed *L. minor* clones, we also sought to estimate their genome sizes. Therefore, we performed k-mer frequency-based genome size estimation using Illumina SR sequencing data. The estimated genome sizes ranged from 318 to 741 Mb (Table 1), spanning a wider range than previous flow cytometry estimates (356–604 Mb)[16]. The median heterozygosity rate was 0.68%, with values ranging from 0.07% to 3.05% (Table 1). The k-mer frequency profiles were grouped into three distinct patterns (Figs. 1A-C; S2): 1) single peak; 2) double peak with the first peak taller; and 3) double peak with the second peak taller. K-mer profiles with two peaks typically indicate heterozygosity or polyploidy, whereas a single peak suggests a homozygous diploid genome[30]. As expected, clones with heterozygosity below 0.2% consistently exhibited a single k-mer peak, while those with higher heterozygosity displayed a secondary peak or hump (Figs. 1A-C; S2). The genomes clustered into three k-mer-predicted average genomes sizes of 330, 514 and 723 Mb that matched with the *L. minor* diploid, *L. ×japonica* triploid, and *L. ×japonica* allodiploid respectively (Fig. 1D). Heterozygosity was almost exclusively associated with the *L. minor* diploid clones, whereas the *L. ×japonica* allodiploid exhibited a single peak and low heterozygosity, despite its k-mer-predicted genome size being double that of the *L. minor* and *L. turionifera* base genome (330 Mb) (Fig. 1D). Since sequencing coverage confirms that the allodiploid clones contain both *L. minor* and *L. turionifera* genomes, explaining the 2x genome size prediction, their extremely low heterozygosity suggests that the k-mer frequency distribution appears haploid-like. This likely results from the high genetic divergence between the parental genomes, which reduces detectable heterozygosity despite the hybrid nature of the genome. These results in conjunction with flow-cytometric genome size determination, chromosome counting and genomic in situ hybridization (see below) indicate that relying solely on k-mer analysis may lead to misinterpretation of the data.

Validating ploidy with smudgeplot

While we established the ploidy levels for all the clones based on coverage depth, we wanted to see if we could recapitulate these results using a k-mer based approach. Therefore, we leveraged the k-mer based Smudgeplot, a computational tool for ploidy inference, to estimate ploidy levels across the presumed *L. minor* clones[31]. The k-mer-based Smudgeplot estimates of ploidy generally agreed with our classifications (Supplementary Data S1;

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Table 1 | Presumed *Lemna minor* clones from the RDSC Collection evaluated in the study with the new species designation (predicted species) based on molecular data

#	clone(#)/ alternative name	*Predicted species*	Predicted ploidy	Continent	Mapping rate (%)	M cov (%)	T cov (%)	Genotype	M/T coverage ratio	K-mer Predicted genome size (Mb)	Heterozygosity(%)	Assembled genome size (Mb)	Flow cytometry (Mb)
1	5500	*L. minor*	DI	EU	99	97	8	MM	>10	318	0.60	308	397@
2	5576	*L. minor*	DI	EU	99	97	7	MM	>10	326	0.60	310	405$
3	6591	*L. minor*	DI	NA	99	97	7	MM	>10	325	0.60	314	406$, 451*
4	7016	*L. minor*	DI	EU	98	98	14	MM	>10	337	0.70	319	409$
5	7022	*L. minor*	DI	EU	99	97	9	MM	>10	325	1.00	330	393$
6	7678	*L. minor*	DI	EU	99	95	7	MM	>10	325	0.20	289	396$, 330@
7	7755	*L. minor*	DI	EU	98	97	11	MM	>10	343	0.60	312	401$
8	7766	*L. minor*	DI	OC	99	97	7	MM	>10	325	0.70	313	397$
9	8417	*L. minor*	DI	EU	99	96	6	MM	>10	323	0.50	308	413$
10	8623	*L. minor*	DI	EU	99	97	10	MM	>10	334	0.60	310	409!, 377*
11	8731	*L. minor*	DI	OC	99	97	6	MM	>10	322	0.70	314	403, 357*
12	8744	*L. minor*	DI	EU	99	96	9	MM	>10	328	0.80	324	399$
13	9253	*L. minor*	DI	EU	99	97	9	MM	>10	324	0.70	NA	399$,356*
19	9345; DWC114	*L. minor*	DI	EU	99	96	6	MM	>10	323	0.60	310	392$
14	9415	*L. minor*	DI	EU	99	96	42	MM	>10	321	0.60	309	408$, 380@, 368*
15	9438	*L. minor*	DI	EU	98	97	12	MM	>10	344	0.70	316	402$, 320@, 394*
16	9441	*L. minor*	DI	EU	98	97	8	MM	>10	352	1.00	306	403$, 373*
17	9482	*L. minor*	DI	EU	99	96	8	MM	>10	324	0.50	NA	404$
18	9495	*L. minor*	DI	EU	99	97	7	MM	>10	327	0.60	312	403$
20	9533	*L. minor*	TR	EU	98	98	12	MMM	>10	326	1.50	424	641$
21	7018	*L. minor A*	DI	AS	97	92	14	MM	>10	330	0.50	302	415$, 570@, 383*
22	7194	*L. minor A*	DI	AF	98	91	11	MM	>10	318	0.30	286	398$
23	7210	*L. minor A*	DI	AF	97	89	15	MM	>10	330	0.10	298	416$, 407*
24	7283	*L. minor A*	DI	AS	96	90	13	MM	>10	338	0.20	299	426$, 300@, 432*
25	7295	*L. minor A*	DI	AF	97	91	14	MM	>10	333	0.70	308	413$
26	7650	*L. minor A*	DI	AF	98	97	12	MM	>10	327	2.50	410	397$
27	7753	*L. minor A*	DI	AF	98	95	14	MM	>10	329	3.10	429	397$
28	8211	*L. minor A*	DI	NA	98	97	13	MM	>10	327	2.50	408	395$
29	8866	*L. minor A*	DI	AS	98	97	12	MM	>10	328	2.50	410	397$
30	7283; DWC123;UTCC270	*L. minor A*	DI	AS	97	89	12	MM	>10	357	0.20	292	426$, 300@, 432*
31	8292#	*L. minor A*	TR	AS	11	91	6	MMM	>10	250	0.50	259	612$
32	7868#	*L. ×japonica*	TR	EU	n.d.	n.d.	n.d.	MMT	n.d.	n.d.	n.d.	n.d.	644$

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Table 1 (continued) | Presumed *Lemna minor* clones from the RDSC Collection evaluated in the study with the new species designation (predicted species) based on molecular data

#	clone(#)/ alternative name	*Predicted species*	Predicted ploidy	Continent	Mapping rate (%)	M cov (%)	T cov (%)	Genotype	M/T coverage ratio	K-mer Predicted genome size (Mb)	Heterozygosity(%)	Assembled genome size (Mb)	Flow cytometry (Mb)
33	7123	*L. ×japonica*	AD	NA	99	95	94	MT	1	741	0.10	609	464$, 414*
41	7405#	*L. ×japonica*	TR	EU	99	96	95	MMT	2	507	2.00	633	460@, 640$
34	7620	*L. ×japonica*	AD	EU	99	95	94	MT	1	741	0.10	610	450$
35	7730; DWC126;UTCC492	*L. ×japonica*	AD	NA	99	95	94	MT	1	729	0.10	610	448$, 411*
36	8434; DWC107	*L. ×japonica*	AD	NA	99	95	94	MT	1	734	0.10	611	460$, 419*
37	8625	*L. ×japonica*	AD	EU	99	94	95	MT	1	711	0.10	NA	427$, 560@
38	8676	*L. ×japonica*	AD	AS	99	95	95	MT	1	709	0.10	608	426$, 376*
39	9580	*L. ×japonica*	AD	EU	99	94	95	MT	1	697	0.10	603	443$
40	7136	*L. ×japonica*	TR	NA	99	97	95	MMT	2	511	2.00	635	626$
42	7436; DWC106	*L. ×japonica*	TR	EU	99	97	95	MMT	2	514	1.90	629	643$, 557*
43	8388	*L. ×japonica*	TR	EU	99	96	95	MMT	2	512	2.00	632	625$
44	8389	*L. ×japonica*	TR	AF	99	96	95	MMT	2	509	1.90	626	625$, 585@
45	8762	*L. ×japonica*	TR	EU	99	96	94	MMT	2	513	1.90	625	644$
46	8784	*L. ×japonica*	TR	EU	99	97	95	MMT	2	514	2.00	635	634$
47	9436; DWC121	*L. ×japonica*	TR	EU	99	96	95	MMT	2	508	2.20	639	641$
48	9437	*L. ×japonica*	TR	EU	99	96	95	MMT	2	514	1.80	619	641$, 760@
49	9439; DWC118	*L. ×japonica*	TR	EU	98	97	96	MMT	2	NA	NA	625	646$, 572*
50	9440	*L. ×japonica*	TR	EU	98	97	96	MMT	2	532	2.00	626	647$, 650@, 578*
51	9486	*L. ×japonica*	TR	EU	99	96	95	MMT	2	514	1.90	623	650$
52	9541	*L. ×japonica*	TR	EU	99	97	95	MMT	2	512	2.00	NA	642$
53	9553; LOT02L	*L. ×japonica*	TR	EU	99	97	95	MMT	2	506	2.00	633	643$
54	EL022	*L. ×japonica*	TR	EU	99	96	95	MMT	2	511	1.80	623	628$, 568*
55	9016	*L. ×japonica*	TR	AS	99	94	97	MTT	0.5	536	1.80	622	670$, 561*
56	9425a	*L. ×mediterranea*	TR	EU	67	95	23	MGG	0.5^	623	1.29	660	733$
57	5635; DWC138	*Landoltia punctata*	DI	NA	30	7	6	NA	NA	371	0.75	349	430$
58	7264	*Landoltia punctata*	DI	EU	29	6	5	NA	NA	368	0.49	345	433$

Predicted species: A, African-clade. From k-mer based pan-genome comparison (Fig. 3) and TBP intron size variation analyses (Fig. 4), these accessions form a distinct subgroup from the other *L. minor* clones.

Predicted ploidy: *DI* diploid, *TR* triploid, *AD* allodiploid.

Continent: *AF* Africa, *AS* Asia, *EU* Europe, *OC* Oceania, *NA* North America.

Genotype: MM, Diploid *L. minor*; MMM triploid *L. minor*; MT, allodiploid *L. minor* × *L. turionifera* (*L. ×japonica*); MTT and MMT *L. ×japonica* triploid; MGG, *L. minor* × *L. gibba* (*L. ×mediterranea*) triploid.

M/T ratio: Ratio of the short-read coverage over the *L. minor* (M) and *L. turionifera* (T) genomes.

Flow-cytometry measurements: Published in Wang et al.[16] (*); Published in Hoang et al.[19] (!); Rutgers (@); IPK ($).

^Clone 9425a was remapped to a concatenated version of *L. minor* × *L. gibba* to establish triploid (MGG; 0.5) coverage.

(#) clone 8292 was identified as *L. minor* A by WGS and TBP, and flow-cytometry suggested it was triploid; clone 7868 was identified as *L. ×japonica* by TBP and flow-cytometry also suggested it was a TR, genome sequencing was not successfully performed (n.d.) due to clone contamination; clone 7405 was identified as *L. ×japonica* by TBP and WGS, but the flow-cytometry estimate by Rutgers (@) was low in contrast to the higher estimate by IPK ($) which is more consistent with triploidy (MMT).

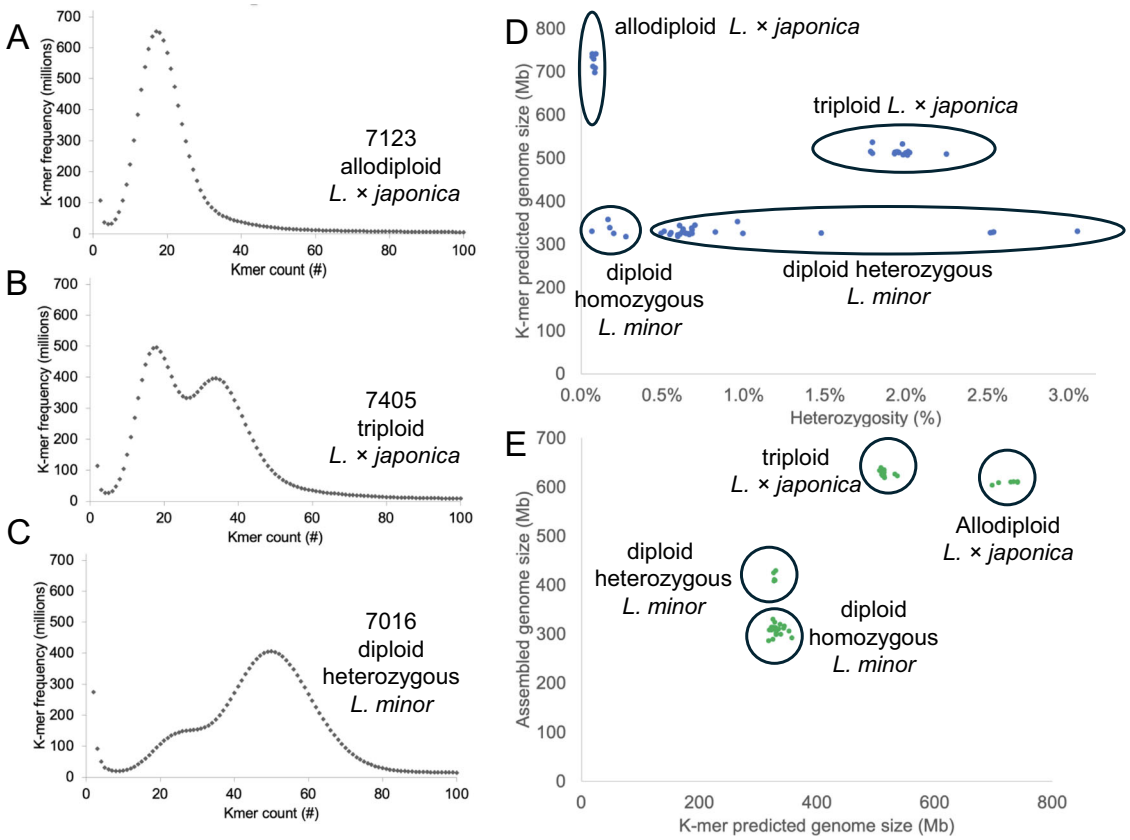


Fig. 1 | Presumed *L. minor* genome feature estimation using Illumina short reads (SR) and k-mer as well as genome assembly analysis. A–C K-mer frequency histograms show distinct genome structure patterns for representative clones across different ploidy levels: **A** Allodiploid (7123) exhibits a single dominant peak, consistent with low heterozygosity; **B** triploid (7405) displays a bimodal distribution, indicative of increased heterozygosity; and **C** heterozygous diploid (7016) shows a distinct double peak, reflecting higher allelic diversity. **D** K-mer-based genome size estimates plotted against heterozygosity (%) reveal four distinct *Lemna* genome classes: homozygous diploids, heterozygous diploids, allotriploids, and allodiploids. The allodiploid genome exhibited an unusual pattern, with a predicted size twice the base *L. minor* genome size of 350 Mb. This is because *L. minor* and *L. turionifera* are genetically divergent enough that their sequences are recognized as distinct in the k-mer frequency histogram, rather than overlapping as would be expected in a more closely related hybrid. **E** Comparison of k-mer-based genome size predictions and assembled genome sizes highlights four genome categories, with triploids exhibiting the largest assembled genome sizes. Species and ploidy designation determined based on the mapping results (Table 1; Supplementary Data S1, S2).

Fig. S3), but there were several important exceptions. For three of the diploid *L. minor* genomes Smudgeplot predicted one as tetraploid (clone 7194) and two as triploid (clones 9533 and 8866). In addition, three of the *L. ×japonica* triploids were predicted to be diploids by Smudgeplot (clones 9436, 9541, and 7436). However, Smudgeplot accurately predicted all of the *L. ×japonica* allodiploid clones as diploids (Supplementary Data S1). Smudgeplot is sensitive to sequencing coverage, and performs best above 15x sequencing depths[31]. At least for the three *L. ×japonica* triploids, the effective haplotype coverage was borderline, so it is possible that the discrepancies observed reflect issues stemming from lower than optimal sequencing depths.

Flow cytometry derived genome size estimates

Flow cytometry is a powerful tool for estimating genome size, providing rapid and quantitative measurements of cellular DNA content[32]. We performed flow cytometry on all of the duckweed clones studied here and compare the results to our sequencing-based genome size estimates, with additional validation by two independent laboratories that maintain these clones (Table 1; Supplementary Data S1). Despite some quantitative differences across labs (Supplementary Data S1), most estimates followed the relative genome size trends observed in the original study[16]. Specifically: 1) Confirmed *L. minor* diploids had genome sizes of ~400 Mb, consistent with the 1 C haploid base genome size; 2) *L. ×japonica* triploids had genome sizes 1.5× larger than diploids (510–650 Mb); 3) *L. ×japonica* allodiploids had genome sizes of >400 Mb, somewhat higher than *L. minor* diploids due to the larger genome of *L. turionifera*[19] (Table 1). We also examined flow cytometry results for clones that were potentially misclassified by Smudgeplot. Clone 9533, initially predicted as a diploid *L. minor* based on k-mer and depth analysis, was identified as triploid by Smudgeplot; a finding that was confirmed by flow cytometry (641 Mb). Clone 7194, classified as a diploid *L. minor* by most methods, was called tetraploid by Smudgeplot but confirmed as diploid by flow cytometry (398 Mb). Two of the three *L. ×japonica* triploids that were incorrectly called diploid by Smudgeplot had flow cytometry estimates supporting triploidy (7436: 557 Mb; 9436: 641 Mb). Flow cytometry results differed between laboratories for clones 7018, EL022, 9437, 9440, and 8625, with discrepancies of roughly one ploidy level. Clone 7405 was consistently identified as triploid *L. × japonica* by all methods except flow cytometry in one laboratory, which indicated diploidy. These differences likely reflect the use of non-identical biological samples under the same clone identifiers and/or the used reference species with assumptions made about their genome sizes. Smaller discrepancies are attributable to variation in reference standards and assumed genome sizes. When observed under identical laboratory conditions, intraspecific genome size differences likely result from long-term clonal propagation. Overall, flow cytometry is a reliable method for genome size estimation when performed under equivalent conditions.

Cytogenetics validate genomics-based results

A robust approach to confirm ploidy status is direct chromosome counting[33]. We selected a subset of clones for chromosomal visualization to validate our genome size and sequencing-based predictions (Fig. 2). As

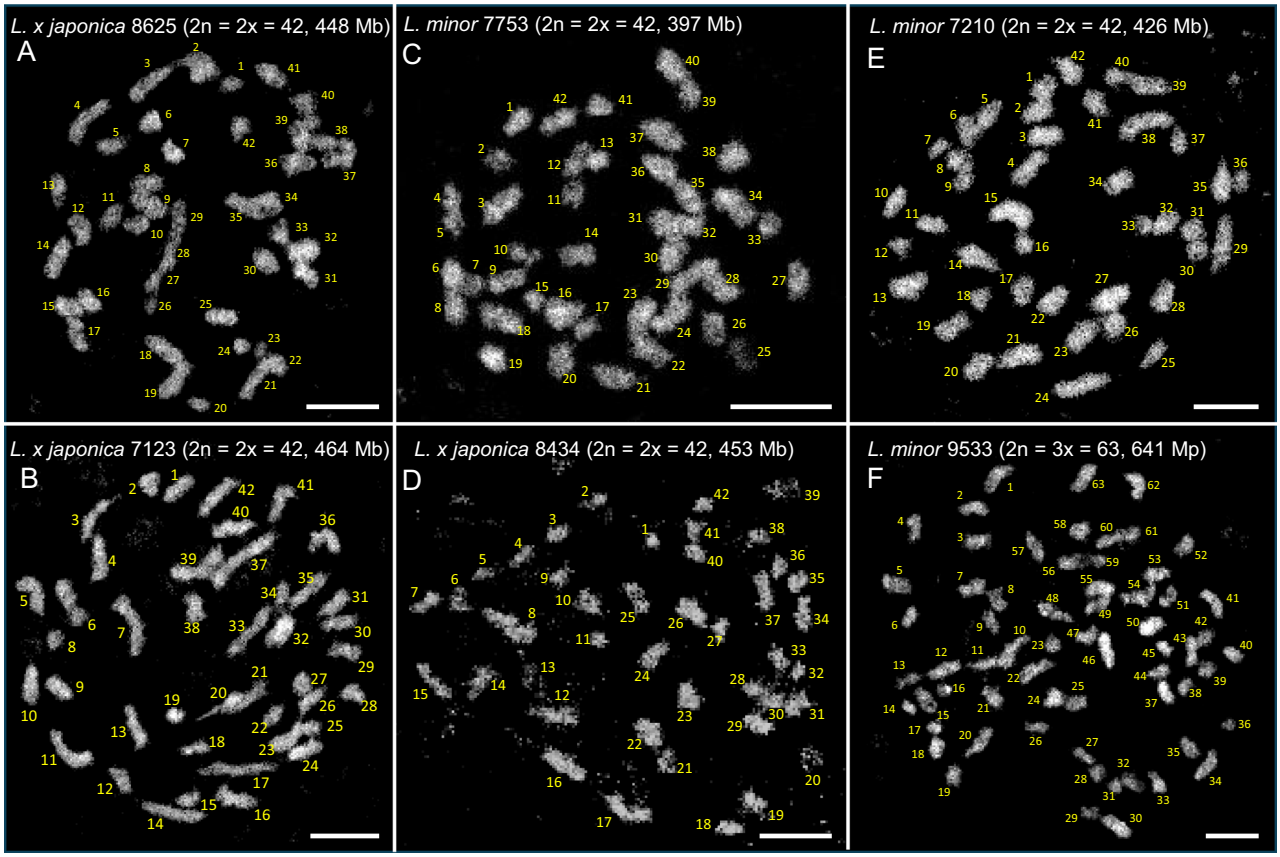


Fig. 2 | Mitotic chromosome spreads with clones of *Lemna*. **A** *L.×japonica 8625* di-haploid; **B** *L. ×japonica 7123* di-haploid; **C** *L. minor 7753* diploid; **D** *L. ×japonica 8434* allodiploid; **E** *L. minor 7210* African-clade diploid; **F** *L. minor 9533* triploid. The yellow numbers simply depict the chromosomes being visualized as they are being counted. Bars = 5 μm.

expected, clones 7210 and 7753, representing both homozygous and heterozygous *L. minor* diploids, had 42 chromosomes (2n = 2x = 42). However, clone 9533, which appeared diploid based on read depth and k-mer analysis but was classified as triploid by flow cytometry and Smudgeplot, was confirmed to have 63 chromosomes (2n = 3x = 63) and provided an example of a triploid *L. minor* clone. Furthermore, clones 8625, 7123, and 8434, all identified as *L. ×japonica* allodiploids, consistently exhibited 42 chromosomes (2n = 2x = 42), confirming their allodiploid status.

Genome assembly to clarify species assignment

De novo genome assembly using Illumina SR typically results in modest-sized contigs, yet it effectively captures gene space and provides valuable insights into genome relationships[30]. Therefore, we de novo assembled the Illumina SRs and found the genomes ranged in quality with N50 lengths spanning from 3 to 21 Kb, which is consistent with assemblies from short reads (Table 1; Supplementary Data S1). The genomes grouped into two size classes at 300–400 Mb and 600 Mb that matched with *L. minor* diploid and *L. ×japonica* allodiploid/triploid respectively (Fig. 1E). Among the predicted diploid *L. minor* clones, five individuals (9533, 8211, 7650, 8866, and 7753) had relatively larger assembled genome sizes (400 Mb) and were also predicted to have the highest heterozygosity (1.5–3.1%). While clone 9533 was subsequently proven to be an autotriploid *L. minor* as described above, the larger assembled genome sizes in the other heterozygous diploids can be attributed to the incomplete collapse of haplotypes, a common issue in SR assemblies. When heterozygosity is high, haplotypes contain sufficient variation to be assembled as separate contigs, leading to inflated genome size estimates. This pattern is further supported by a strong negative correlation ($r = -0.8$) between N50 contig length and heterozygosity (Fig. S4), indicating that higher heterozygosity results in shorter contigs and larger total assembly sizes due to partial haplotype duplication. The *L. ×japonica* allodiploid/triploid assemblies were approximately twice the size of the diploid *L. minor* genomes, consistent with separate assembly of both sub-genomes. This interpretation is reinforced by mapping these assemblies back to the *L. ×japonica* 8627 reference genome[25], which revealed a clear separation of subgenomic sequences (Fig. S5). The genome assemblies estimated that the base *L. minor* genome size was ~350 Mb in contrast to the value estimated by flow cytometry (~400 Mb), which could reflect the fact that SR assembly does not generally assemble repeat sequences like centromere and transposable elements well. The larger variation observed previously by flow cytometry[16] was most likely due to *L. ×japonica* triploids.

Reference-free super-pangenome

A super-pangenome extends the traditional pangenome concept by incorporating multiple species within a genus or across related taxa, rather than being confined to a single species[34]. We leveraged the highest-quality 38 SR de novo genome assemblies in our dataset to construct a reference-free super-pangenome (Fig. 3); we also leveraged published chromosome-scale genomes to place the super-pangenome in the context of *L. ×japonica*, *L. turionifera* as well as published genomes of other duckweed genera[10,23,25–27] (Fig. S6). Using PanKmer[35], we built a shared k-mer matrix to assess genome-wide relationships across the diploid *L. minor* and *L. ×japonica* allodiploid and triploid clones (Fig. 3). The reference-free super-pangenome matrix revealed three primary clusters, with a clear outlier grouping of *Landoltia punctata* (clones 5635 and 7264). Additionally, *L. ×mediterranea* (clone 9425a) branched separately with the known diploid *L. minor* clone Lmin5500[17], supporting its distinct hybrid status. A particularly useful validation came from clones DWC123 and 7283, which were descendants of the same clone independently maintained by two stock centers for over at least two decades. Their close clustering in the super-pangenome matrix

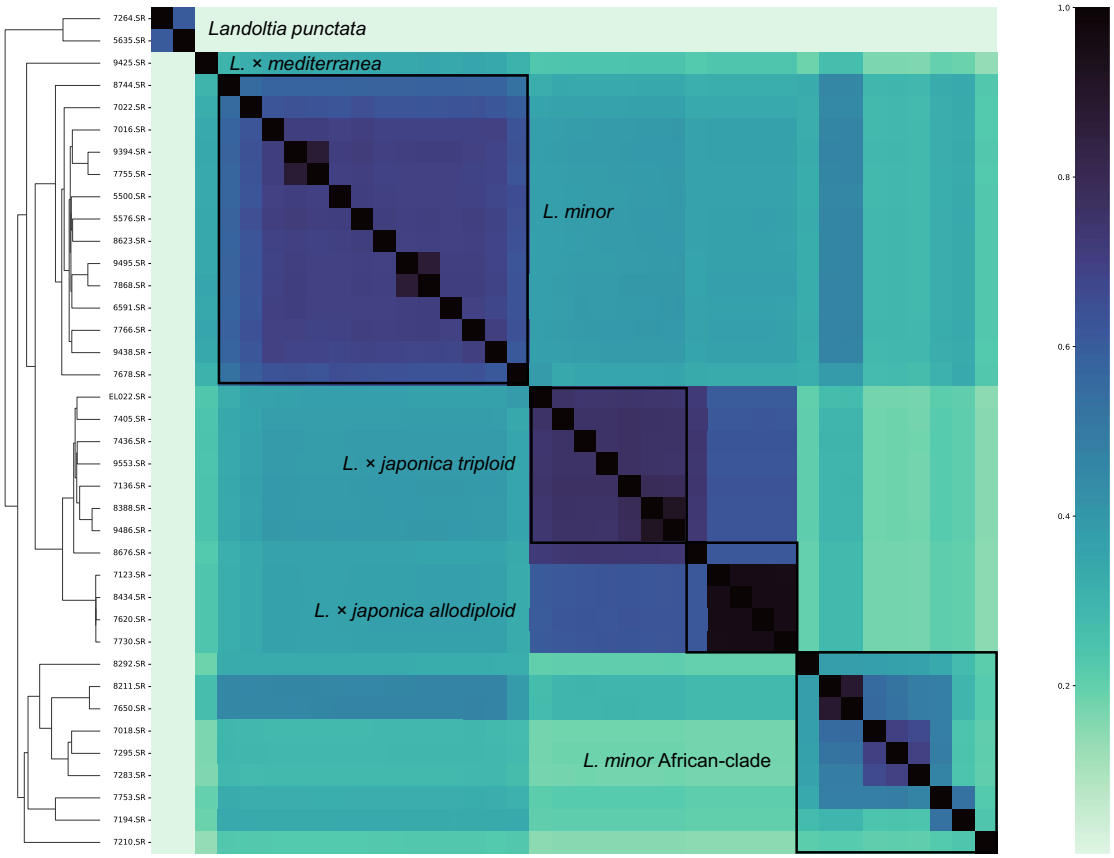


Fig. 3 | Reference-free super-pangenome based on Illumina short read (SR) arranged presumed *L. minor* genomes into species and ploidy classes based on k-mer distance. A Jaccard super-pangenome matrix was generated with Illumina short read (SR) based genome assemblies using PanKmer ($K = 31$). Darker color corresponds to less k-mer distance, while lighter color corresponds to more k-mer distance. *Landoltia punctata*, *L.* ×*mediterranea*, *L. minor* diploids, *L.* ×*japonica* triploids, *L.* ×*japonica* allodiploids, and *L. minor* African-clade are boxed.

confirms the reliability of our approach in capturing genomic relationships.

The three main clusters in the super-pangenome included two on either side of the matrix that contained the diploid *L. minor* clones, while the third cluster in the middle contained the *L.* ×*japonica* allodiploids and triploids (Fig. 3). The top left *L. minor* diploid cluster included the published Lmin5500 clone[17], which suggested that the bottom right *L. minor* diploid cluster might represent a distinct sub-class. Moreover, the top left *L. minor* diploid cluster shared more k-mers with the *L.* ×*japonica* allodiploid/triploid cluster than the other diploid cluster; this indicates that the diploid clusters may represent different clades as well as suggesting that the *L. minor* diploids from the top left cluster were the sub-class that contributed to the *L.* ×*japonica* hybrids. When we included all published duckweed chromosome-scale genomes in the super-pangenome, the *L.* ×*japonica* allodiploid/triploid cluster further supported the hybrid nature with *L. turionifera* and suggested that the *L.* ×*mediterranea* hybrid 9245a may have shared k-mers with clone 7210 from the *L. minor* African-clade (Figure S6). One potential explanation is that the *L. minor* parental genome contributing to the *L.* ×*mediterranea* hybrid has common ancestry with the 7210 African lineage. However, currently, there is insufficient information to resolve the relationship between *L.* × *mediterranea* and clone 7210. In any case, our approach enabled a reference-free comparison of genome structure and hybridization patterns, which enhanced our ability to differentiate sub-classes within *L. minor*, track hybrid origins, and explore genome evolution at a broader taxonomic scale. These findings demonstrate that super-pangenome matrices provide a powerful genome-wide approach for rapidly assessing relationships between clonal populations within a species and even across genera.

Tubulin intron-based polymorphism (TBP) validation

TBP calling is another approach that has been utilized to clarify the relationship between *Lemna* species as well as to identify *L.* ×*japonica* as a hybrid derived from *L. minor* and *L. turionifera*[13,20,22]. All the presumed *L. minor* clones (except the *Landoltia* and *L.* ×*mediterranea* clones) were validated using the TBP marker; both cluster as well as PCA analyses were consistent with three groupings like the super-pangenome matrix (Fig. 4; Supplementary Data S1). The top left cluster from the super-pangenome matrix overlapped with the true *L. minor* diploid clones that are similar to Lmin5500 (*L. minor* 5500-like; Fig. 4). The center super-pangenome cluster overlapped with the *L.* ×*japonica* cluster as predicted. TBP analysis suggested clone 7868 was *L.* ×*japonica* triploid, with likely MMT composition, consistent with the flow cytometry-based genome size of 644 Mb (Table 1). However, mapping of the SR sequence reads indicated a mix up of clones was likely to have taken place in the sequencing stage. The third TBP cluster that was distant compared to the other two clusters (*L. minor* African-clade), overlapped with the lower right cluster of *L. minor* diploids in the super-pangenome analysis, which we also found to be more distant in the k-mer matrix. All these clones come from Africa or several Middle Eastern countries, such as Iran and Turkey. Therefore, we have termed this subclass the "African-clade". Considering the apparent diversity of this cluster in the super-pangenome matrix, the "African-clade" could represent a different species or subspecies, or even further hybrids, but distinct from the recently identified *L.* ×*mediterranea* clones[14,28].

Confirmation of the hybrids by genomic in situ hybridization (GISH)

To confirm the ploidy status and genome components of *L.* ×*japonica* triploids and allodiploids, we performed GISH studies for select clones. Using species-derived probes generated from *L. minor* (M) and *L.*

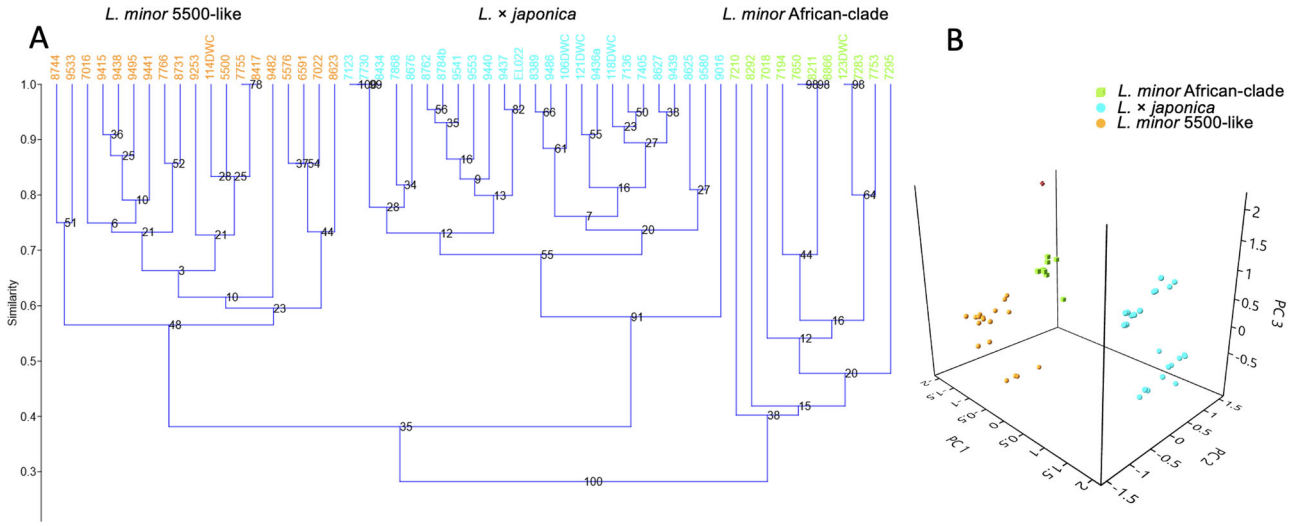


Fig. 4 | TBP analysis confirms *L. minor* and the hybrid *L. ×japonica*, as well as suggests a more distantly related *L. minor* African-clade. A Jaccard Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree based on the β-tubulin intron size variation. **B** PCA plot of the relationship between *L. minor* clones using *Landoltia punctata* as outgroup (red symbol).

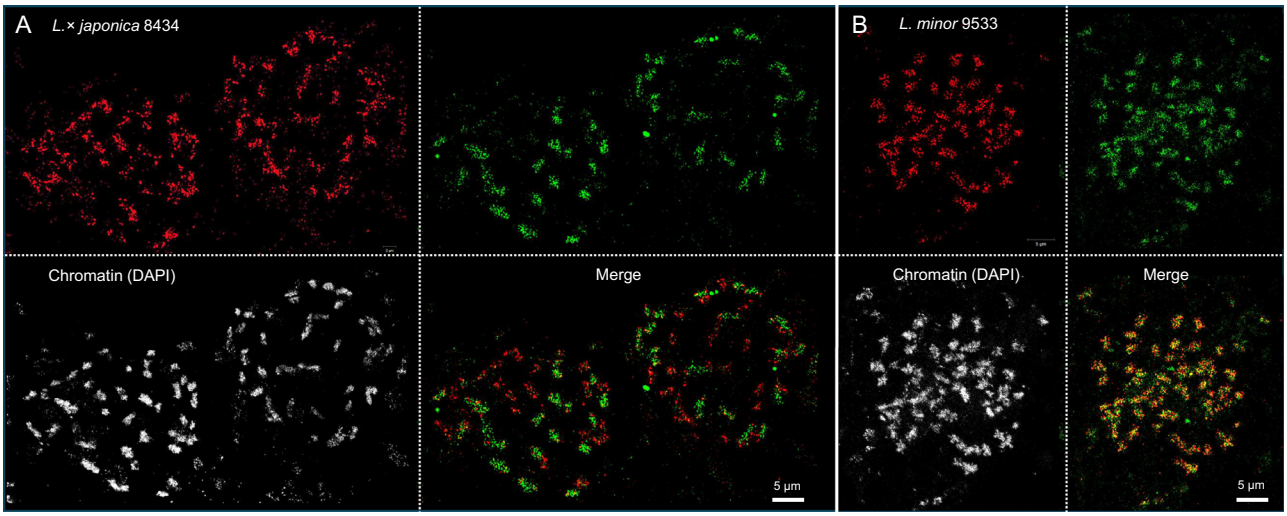


Fig. 5 | Genomic in situ hybridization (GISH) reveals contrasting chromosome compositions between an interspecific hybrid and an autotriploid *Lemna* accession. GISH on somatic metaphase chromosomes of the allodiploid *L.×japonica* 8434 (**A**) and the autotriploid *L. minor* 9533 (**B**). GISH was performed with fragmented genomic DNA of *L. minor* (red) and *L. turionifera* (green) labeled with Cy3-dUTP or Alexa Fluor 488-5-dUTP, respectively. These probes are hybridized to chromosome spreads from different duckweed genotypes as described in Methods. The weaker green signals found in (B) result from the low percentage of sequence homologies between *L. minor* and *L. turionifera* genomes and thus hybridize to the same chromosomes as the red signals producing yellow signals (Merge). In (A), the two signals are largely distinct since the two probes hybridize preferentially to different sets of chromosomes and thus contain very few yellow signals, if at all.

turionifera (T) genomes, we visualized the sub-genomes at the chromosome level (Fig. 5). For the *L. ×japonica* allodiploid clone 8434 (Ljap8434), GISH confirmed a diploid chromosome number (Figs. 2D; 5A), reinforcing its classification. Clone 9533, originally identified as diploid based on k-mer analysis and read depth, was later predicted as triploid by Smudgeplot and flow cytometry. Our GISH analysis validated the triploid classification (2n = 3x = 63; 641 Mb/1 C) by demonstrating that all chromosomes were predominantly labeled by *L. minor* DNA (Fig. 5B). This confirms that 9533 is a *L. minor* autotriploid (see also Fig. 4), not a hybrid, explaining why it initially clustered with diploid clones. Notably, 9533 and 8292 are the only confirmed *L. minor* triploids in our dataset. Additionally, GISH confirmed that clone 8627 is a *L. ×japonica* triploid (2n = 3x = 63)[25], further supporting our ploidy classifications. These results reinforce the importance of combining sequencing-based and cytogenetic methods to accurately classify ploidy and genome composition, particularly in hybrid species.

Summary of presumed *L. minor* clone identity

Integrating results from multiple orthogonal approaches and by independent laboratories, we reclassified the 58 presumed *L. minor* clones based on their species identity and ploidy level (Table 1; Supplementary Data S1). We found that 53% were *L. minor* clones that separated into two sub-classes (5500-like and 7210-like), and that clones 9533 and 8292 were the only two *L. minor* triploids in this collection studied. However, 47% of the collection was mis-called. Most of them were reassigned to *L. ×japonica* allodiploid (12%), and allotriploid (29%) hybrids that have derived from hybridization with *L. turionifera* (Table 1; Supplementary Data S1). We only found one clone (7425a) in this collection (1.7%) that was a hybrid between *L. minor* and *L. gibba* (*L. ×mediterranea*). Finally, we identified two clones (3.4%) that were from the monospecific genus *Landoltia*, suggesting that confusion in assigning clone identity across the *Lemna* genus extends to other genera. These findings underscore the importance of integrating multiple genomic

Table 2 | Growth measurements across the *Lemna* inter- and intraspecific hybrids and diploids

	RGR (day^{-1})	DT (days)	RY (wk^{-1})	MA (cm^2)
Allodiploid (*L.* ×*japonica*) *N* = 4	0.48 ± 0.01	1.46 ± 0.04	30.74 ± 2.54	1.14 ± 0.04
Diploid African-clade (*L. minor* A) *N* = 4	0.37 ± 0.02	2.08 ± 0.09	16.6 ± 1.96	1.26 ± 0.08
Diploid (*L. minor*) *N* = 7	0.41 ± 0.01	1.71 ± 0.06	19.72 ± 1.55	0.96 ± 0.07
Triploid (*L.* ×*japonica*) *N* = 3	0.46 ± 0.02	1.53 ± 0.06	26.9 ± 3.44	1.52 ± 0.06
Autotriploid (*L. minor*) *N* = 2	0.42 ± 0.02	1.71 ± 0.07	22.24 ± 2.72	1.78 ± 0.05

Relative growth rate (RGR; day^{-1}), doubling time (DT; day), relative weekly yield (RY; week^{-1}), and mean frond area (MA; cm^2) are determined as described[38]. Standard deviations are shown for each of the measurements. The number of lines used for each of the groups are shown as *N* and the specific clone numbers can be found in Supplementary Data S3. ANOVA statistical analyses for significance can be found in Supplementary Data S5.

and cytogenetic tools for accurate species and ploidy classification, as misidentifications remain common in collections of morphologically similar duckweed species.

Polyploids may provide growth advantage in some environments but not in others

Polyploidy has been hypothesized to enhance vigor and environmental adaptability, whereas diploids are often thought to be specialized for more narrow ecological niches[36]. In *Lemna*, both interspecific and intraspecific hybrids may benefit from increased growth rate and environmental flexibility due to heterosis and genomic buffering[37]. We measured growth parameters for 20 selected clones that have been genomically resolved in this study, which included diploids and polyploids from the *L. minor* complex: relative growth rate (RGR), doubling time (DT), relative yield (RY), and mean frond area (MA)[38]. Both *L.* ×*japonica* allodiploid and triploid hybrid clones exhibited superior growth performance across most of these traits when compared with the diploids. Allodiploids outperformed diploids with reasonable significance (p-values of <0.5) in DT, RY, and RGR, but showed the smallest effect on MA (Table 2; Supplementary Data S3, S5). Although the sample number is the smallest for the *L. minor* triploids ($N = 2$), since these are the only two of this genotype in our collection, the triploid *L. minor* clones (9533 and 8292) displayed the largest mean area (MA, $P < 0.05$) among all classes. The *L.* ×*japonica* triploids (N = 3) comes in a close second for largest average MA and together with the *L. minor* triploids provided a stronger case ($N = 5$) that a size advantage in MA is associated with triploidy in comparison to diploids ($N = 15$). In contrast, diploid *L. minor* clones were the most variable and exhibited lower overall vigor, reinforcing the hypothesis that polyploidy and hybridization can contribute to greater robustness and growth potential.

Polyploids have also been hypothesized to exhibit greater vigor and adaptability across a wider range of environmental conditions[39]. In a second independent exploratory experiment we examined this in the *L. minor* complex by measuring relative growth rate (RGR), relative yield (RY), and doubling time (DT) for one representative clone from each class (*L. minor* diploid, *L.* ×*japonica* allodiploid, and *L.* ×*japonica* triploid) under five different light and temperature conditions at 7 and 14 days (Figure S7; Supplementary Data S4, S5). Growth was assessed at two time points since earlier growth rates tend to be faster and more linear with less plant crowding and higher nutrient availability. Significant differences ($P < 0.01$) were observed for both conditions and clone effects on RGR and RY, while DT showed no significant associations (Figure S7; Supplementary Data S4, S5). Under control conditions, *L.* ×*japonica* allodiploid and triploid grew 24% and 35% faster than the *L. minor* diploid. A larger growth advantage emerged under low-light conditions, where the hybrids exhibited 81% (allodiploid) and 51% (triploid) higher growth rates compared to the diploid. Importantly, under higher temperature or high light stresses, the allodiploid and triploid hybrids both showed less growth compared to the diploid *L. minor* plants. These findings suggest that while polyploidy or hybridization may confer a growth advantage under specific environmental conditions, particularly low-light stress, they could be less tolerant to more extreme abiotic stressors such as high temperature or excess light. However, due to the small number of sample size in this exploratory study, future studies with larger numbers of these genotypes from our collection of genome-resolved clones from the *L. minor* complex would be necessary to confirm and extend these initial observations.

Discussion

Lemna minor, also known as Common Duckweed, is widely regarded as the most abundant and broadly distributed duckweed species on Earth[11]. In this work, we leveraged a multi-pronged approach with several orthogonal methods to resolve the species and ploidies of 58 previously presumed *L. minor* clones from the RDSC collection (see chart in Figure S8 for a summary of our work-flow). Our results re-classified these *L. minor* clones into: 1) two classes of diploid *L. minor* clones that includes a grouping we propose to designate as the African-clade (*L. minor A*); 2) allodiploid and triploid *L.* ×*japonica* (*L. minor* × *L. turionifera*) hybrids; 3) one triploid *L. minor* × *L. gibba* (*L.* ×*mediterranea*); and 4) two clones from the *Landoltia* (7264, 5635) genus. In addition, WGS revealed two *L. minor* autotriploid clones (8292, 9533), one of which was validated by GISH and TBP (9533) (Figs. 4 and 5) and the other (8292) by TBP. Moreover, now that we could clearly group the clones by species and ploidy, an initial study showed that the *L. minor* related triploids and hybrids could display growth advantages over the *L. minor* diploids under ambient conditions but not under some abiotic stressors such as excess light or high temperature. These results set the precedent for proper characterization and assignment of future clones added to the collection as well as providing a well-curated collection to enable more in-depth research to determine how polyploidy may impact duckweed growth and population dynamics.

Our analysis revealed some discrepancies between clones from two different laboratories at the U.S. (New Jersey) and Italy (Milan), a phenomenon also observed in the model plant *Arabidopsis thaliana*. Notably, even the widely used reference line Columbia-0 (Col-0) has been shown to accumulate distinct genomic variations when maintained in separate labs[40]. For instance, we found that flow-cytometry results differed by lab for five clones (7018, EL022, 9437, 9440, and 8635), most likely reflecting either experimental mislabeling or the same accession number representing different biological samples (Supplementary Data S1). However, the two clones (DWC123 [UTCC270], and 7283) that were separated by at least two decades in two different collections, but originated from the Landolt Duckweed Collection, were exactly the same (Table 1; Fig. 3). These results highlight that in order to get reliable results, multiple approaches including direct ones (e.g. GS and GISH) in addition to probabilistic ones (e.g. k-mer based methods), are essential, in particular when looking at species complexes like *L. minor* where polyploidy and interspecific hybrids appear to be rather common.

For duckweeds, a puzzling phenomenon remains: triploids and interspecific hybrids, both of which arise only through sexual reproduction, appear to occur relatively frequently in populations that predominantly propagate asexually[14,15,22]. Polyploidy predominantly arises either by fusion of unreduced gametes or after spontaneous chromosome doubling, resulting in allopolyploids if allodiploid hybrids were involved[41]. Triploids appear after fusion of a reduced with an unreduced gamete and can be maintained only by asexual propagation, because irregular meiotic chromosome segregation does not yield viable gametes, as it was recently confirmed in

Lemna hybrids[29]. The apparent loss of genes involved in the small RNA-mediated triploid block pathway as well as of genes that suppress the formation of unreduced gametes seem to enable the frequent occurrence of stable triploid populations in *Lemna*[25]. Moreover, the predominantly asexual propagation habit of duckweeds would be consistent with the avid viability of triploids arising in duckweeds. We found that 41% (24/58) of the previously curated *L. minor* strains (based predominantly on morphological basis) in the RDSC collection were a mixture of *L.* ×*japonica* with 71% (17/24) of those being triploid and 29% (7/24) allodiploids (Table 1; Supplementary Data S1). A similar situation was found for presumed wild *L. minor* clones collected across natural populations in Switzerland where over 50% were later identified as *L.* ×*japonica* triploids by WGS[24]. Their low genetic variability indicated that triploids may emerge, clonally multiply and exploit specific environments. Similarly, among wild presumed *L. minor* clones from Eastern Europe, molecular markers were used to show that *L.* ×*japonica* was prominent[20,21]. Thus, sexual hybrids including triploids are more common than previously thought in nature, suggesting that triploid *L.* ×*japonica* may confer some level of growth advantage over its diploid parental clones in particular habitats. While our data from our growth experiments in this work appears to confirm this hypothesis, more in-depth analysis with larger number of isolates would be necessary to strengthen these preliminary results and conjectures (Table 2; Fig. S7).

Duckweeds exhibit a wide range of genetic diversity and reproductive strategies that influence their adaptability to different environments. Some species, such as *Spirodela polyrhiza*, maintain large population sizes but low sequence variations[42], which suggests that even minor genetic advantages may enhance clonal propagation and facilitate the colonization of new micro-environments. In contrast, some wild populations of presumed *L. minor* have been found to harbor 4 to 20 distinct genotypes per site, reflecting extensive genetic diversity that could aid in local adaptation[43]. Supporting this, *L. minor* exhibits twice as much genetic variation in fitness per site compared to other species, consistent with strong purifying selection acting to counteract environmental fluctuations[44]. While hybrids (allodiploids and triploids) were not explicitly resolved in these previous studies, their presence may contribute to local adaptation and population dynamics. Our initial findings align with this framework, as hybrid and polyploid genotypes outperformed diploids under ambient conditions but could have reduced performance under certain abiotic stressors such as higher temperatures or excess light (Fig. S7; Table 2; Supplementary Data S3-5). Although the growth advantage of triploids appears modest in our study (yet significant), a 5% increase in relative growth rate (RGR) may allow a particular clone to rapidly outcompete and dominate a population over time. In this model, even a small number of diploid or triploid clones that persist under different conditions could act as a genetic reservoir to revert the population to its more advantageous genotype upon significant change in the environment to outcompete other macrophytes.

In conclusion, our comprehensive characterization of presumed *L. minor* clones from the RDSC collection underscores that these clones form a "*L. minor* complex" that includes *L. minor* diploids and autotriploids, as well as allodiploids and reciprocal triploids from interspecific hybrids with either *L. turionifera* or *L. gibba* species[14,25]. Rather than a uniform species, the *L. minor* complex is thus far more genetically diverse and dynamic than previously recognized and its naming would be consistent with previously suggested treatment for populations that combine unisexual reproduction, interspecific hybrids and ploidy variants to generate diversity[45]. Similarly, a "*Lemna aequinoctialis* complex" has recently been described[15], suggesting that the *Lemna* genus may exploit its environment through various interspecific hybrids. Thus, sexuality plays a more pivotal role in duckweed evolution than once assumed. These findings provide clues to how polyploidy and hybridization may promote ecological success and genetic diversity in duckweeds that predominantly grow by asexual propagation. They also created a knowledge base and genetic resource for future work by the community to more systematically characterize their evolutionary dynamics, taxonomy, and facilitate biotechnological application of duckweeds.

Methods

Duckweed clones

Lemna minor clones were received from the Rutgers Duckweed Stock Collective (RDSC; http://www.ruduckweed.org/). Clones were checked for sterile conditions and if they were not sterile they were re-sterilized per the RDSC protocol. 58 clones were grown under 12 h light and 12 h dark at 22 °C. The collection location and all associated information are reported in Supplementary Data S1.

DNA extraction for genome sequencing

Clones were collected, patted dry to remove excess water and flash frozen in liquid nitrogen. Frozen tissue was ground in a mortar and pestle under liquid nitrogen. HMW DNA was isolated using a modified BOMB protocol (#6.4; https://bomb.bio/protocols/). DNA quality and quantity were assessed on a bioanalyzer and HMW status was confirmed on an agarose gel.

Genome sequencing

Illumina 2 × 150 bp paired end reads were generated for genome size estimates, short-read genome assembly and polishing genome long read assemblies. Libraries were prepared from HMW DNA using NEBnext (NEB, Beverly, MA) and sequenced on the Illumina NovaSeq (San Diego, CA). Resulting raw sequence was only trimmed for adaptors, resulting >60X coverage of the diploid *L. minor* genome (350 Mb).

Variation estimation by read mapping

Illumina paired end reads were mapped to the *L. minor* diploid reference genome (Lmin5500)[17], as well as the updated *L. minor* 9252 and *L. turionifera* 9434 genomes[25] using minimap2[46]. Clone 9425a was also mapped to a concatenated version of *L. minor* 9252 and *L. gibba* 7742a. Mapping rates were summarized in Supplementary Data S1, S2. Resulting sorted bam files were analyzed for mapping statistics using samtools[47] and variants were called using samtools mpileup[48] and vcftools[49].

Genome size estimation by k-mer frequency

K-mer ($k = 31$) frequency was estimated with Illumina paired end reads (2 × 150 bp) using Jellyfish (v2.3.0)[50] and analyzed using in house scripts and GenomeScope and GenomeScope2[31,51]. Genome size, heterozygosity and repeat content were first estimated with GenomeScope[51], which reports haploid genome size (Table 1; Supplementary Data S1). Ploidy was estimated using GenomeScope2 and smudgeplot[31] (Table 1; Supplementary Data S1).

Short read genome assembly

Illumina paired end reads were assembled with Spades (v3.14.0) with standard settings[52]. Resulting contigs were mapped to the *L. minor* reference genome (Lmin5500) to estimate completeness and statistics were determined (Table 1; Supplementary Data S1).

Reference-free pangenome (PanKmer)

The short read-based genome assemblies were used to construct a pangenome using PanKmer[35]. Briefly, a 31-mer index was constructed using PanKmer[35] with default parameters using the short read Illumina assemblies. Jaccard and ANI similarities for all assemblies in both indexes were calculated and visualized using PanKmer by setting the clustermap metric parameter to "--metric jaccard" and "--metric ani", respectively.

Flow cytometry (FC) genome size estimates

At Rutgers. The methods used to prepare the nuclei suspensions were based on the protocol for estimation of nuclear DNA content in plants using flow cytometry[53]. Sixteen clones of *L. minor* and four additional plant species of known ploidy, *Spirodela polyrhiza* (158 Mb), *Arabidopsis thaliana* (134 Mb), *Brachypodium distachyon* (272 Mb), and *Fragaria x ananassa* (800 Mb), were analyzed. These species, along with two of the *L. minor* strains known to be diploid, were used as standards to aid in ploidy estimation of the remaining fourteen *L. minor* varieties. For each strain and standard, 120 mg of fresh tissue was harvested and placed in a petri

dish with 4 mL of Galbraith's buffer, a solution consisting of 45 mM $MgCl_2$, 20 mM MOPS, 30 mM sodium citrate, and 0.1% (vol/vol) Triton X-100. The tissue was gently chopped into small pieces with a disposable razor blade to release nuclei. After the homogenate was filtered through a 40 µm cell strainer, the filtrate was evenly distributed into four micro-centrifuge tubes. Propidium iodide was used to stain the DNA; 50 µL each of 1 mg/mL PI and 1 mg/mL RNase A were added to each of three tubes, the fourth left unstained to act as the control. The samples were incubated on ice for about 15 min and gently shaken periodically before analysis through the flow cytometer (BD/Accuri C6+ Flow Cytometer). The flow cytometer was set to collect data on 10,000 events, with the minimum core size and flow rate due to the small size of the nuclei. The selected threshold was 80,000.

At the IPK. The genome size of *Lemna* clones was determined following the procedure according to Dolezel et al.[53] using the CyStain PI Absolute P reagent kit (Sysmex-Partec) according to the manufacturer's instructions. Fresh frond tissue was co-chopped with fresh leaf tissue of one of the internal reference standards, either *Raphanus sativus* 'Voran' (Gatersleben Gene bank accession: RA 34; 1.11 pg/2 C) or *Lycopersicon esculentum* 'Stupicke Rane' (Gatersleben Gene bank accession: LYC 418; 1.96 pg/2 C). The nuclei suspensions were measured either on a CyFlow Space flow cytometer (Sysmex-Partec) or on a BD Influx cell sorter (BD Biosciences). An example for the gating strategy used is shown on Fig. S9. Each clone was measured at least four times on two different days.

Tubulin intron-based polymorphism (TBP) analysis

The TBP amplification protocol and data analysis were performed, from 30 ng of total genomic DNA, according to the procedure described[54]. The polymorphisms in length of both intron regions (I and II) of the ß- tubulin genes were scrutinized, and each DNA sample was independently analyzed twice.

Growth rate and frond area estimation

For the experiments presented in Table 2 and Supplementary Data S3, clones for relative growth rate (RGR; day^{-1}), doubling time (DT; day), relative weekly yield (RY; $week^{-1}$), and mean area (MA; cm^2) estimation were acclimatized by pre-cultivation for four weeks under axenic conditions in a climate cabinet (PK-520, poly klima GmbH, Freising, Germany) under continuous white light (100 µmol m-2 s-1 PAR) and constant temperature of 25 °C ± 1 °C. The cultivation medium (*N* medium) was changed weekly to avoid nutrient deficiency[55]. For the main experiment, a colony consisting of three to four fronds was placed in a plastic cup containing 40 ml of N medium. After seven days, the number of fronds was recorded again and the relative growth rate was calculated as $RGR = (\ln x_{t7} - \ln x_{t0}) / (t7 - t0)$[38]. The frond area was measured as the mean value of three single fronds per clone in six replicates using a digital microscope (VFX 7000, Keyence, Neu-Isenburg, Germany).

Duckweed growth rate estimation under differential conditions

To compare potential impact of ploidy and hybridization on stress response (Fig. S7 and Tables S4, 5), *L. minor* clones were obtained from RDSC (Rutgers Duckweed Stock Collection) at Rutgers the State University of New Jersey, New Brunswick, NJ, USA. The clones were maintained on 0.5X Schenk-Hildebrand medium with 0.8% w/v agar and 0.3% sucrose with the addition of 100 mg/L cefotaxime. The clones were kept at 25 °C under illumination of 150 µmol $m^{-2}s^{-1}$ light (16 h light/8 h dark) using a Caron PM-7304-50 growth chamber. Five frond colonies of *L. minor* clones from stock cultures were grown for 2 weeks in a solution containing 0.5X Schenk-Hildebrand medium and 0.3% sucrose. For growth measurement, ~100 mg fresh fronds of each clone were then transferred to a 177 ml glass jar containing 50 ml of fresh 0.5X Schenk-Hildebrand medium and 0.3% sucrose solution. The experiment was carried out in 3 replicates with samples collected at days 7 and 14. Tissues of *L. minor* clones were harvested and dried overnight at 55 °C in 1.5 ml Eppendorf tubes and the growth rates measured (Relative Growth Rate [RGR]; day^{-1}), Doubling time (DT; day), Relative yields (RY; week−1) according to procedures as described[38]. For experiments under "control" conditions, the *L. minor* clones were maintained at 25 °C under illumination of 150 µmol $m^{-2}s^{-1}$ light (16 h light/8 h dark). Otherwise, temperature and light intensity were changed as described for the stressor experiments to examine effects by shifts in these environmental parameters.

Trait mean value analysis

A one-way analysis of variance using Welch's ANOVA test was performed to test whether ploidy had a significant effect on each measured trait. Following significant ANOVA results, Games-Howell post-hoc tests were conducted to identify pairwise differences between ploidy groups. Statistical analyses were performed in Python using the pingouin (0.5.5) library.

Treatment analysis

A two-way analysis of variance using ANOVA test was performed to test whether ploidy and experimental treatment had a significant effect on each measured trait. Post-hoc tests using Tukey's HSD were conducted to identify specific differences between ploidy groups. Statistical analyses were performed in Python using the pingouin (0.5.5) and statsmodels (0.14.2) libraries.

Chromosome counting

The fronds were grown in liquid nutrient medium under 16 h white light of 100 µmol m^{-2} s^{-1} at 24 °C[55]. The mitotic chromosome spreading was carried out as described[56]. Fronds were treated in 2 mM 8-hydroxylquinoline, fixed in fresh 3:1 absolute ethanol: acetic acid, softened in PC enzyme mixture [1% pectinase and 1% cellulase in Na-citrate buffer, pH 4.6], macerated and squashed in 45% acetic acid. After freezing in liquid nitrogen, chromosome spreads were treated with pepsin [50 µg/ml in 0.01 N HCl], post-fixed in 4% formaldehyde in 2x SSC [300 mM Na-citrate, 30 mM NaCl, pH 7.0], rinsed twice in 2x SSC, 5 min each, dehydrated in an ethanol series (70, 90 and 96%, 2 min each) and air-dried. Chromosome counting was performed within different slices of 3D-SIM image stacks[19].

DNA extraction for genomic in situ hybridization

For each sample, 0.3 g of fresh and healthy fronds were harvested and cleaned in distilled water, put into a 2 ml Eppendorf tube with two metal balls, frozen in liquid nitrogen, and ground by a ball mill mixer (Retsch MM400). The genomic DNA of the studied species was isolated using the DNeasy Plant Mini Kit (cat. nos. 69104-Qiagen). DNA was eluted by 200 µl buffer AE and quality checked by electrophoresis. Genomic DNA was sonicated before labelling.

Probe preparation

Sonicated genomic DNA (1 µg) was labeled with Cy3-dUTP (GE Healthcare Life Science) or Alexa Fluor 488-5-dUTP (Life Technologies) by nick-translation, then precipitated in ethanol[57] with sonicated unlabeled DNA of the other presumed parental species as carrier DNA in excess. Probe pellets from 10 µL nick translation product for GISH probes were dissolved in 100 µL hybridization buffer [50% (v/v) formamide, 20% (w/v) dextran sulfate in 2× SSC, pH 7] at 37 °C for at least 1 h. The ready-to-use probes were stored at −20 °C.

GISH (Genomic in situ hybridization)

Probes were pre-denatured at 95 °C for 5 min and chilled on ice for 10 min before adding 20 µL probe per slide. Two-rounds of GISH with alternatively labeled genomic probes of the presumed parental species were performed to investigate the distribution of the corresponding probe signals on the chromosome complement of the tested clones as described[19].

Super-resolution microscopy

To analyze the ultrastructure and spatial arrangement of signals and chromatin at a lateral resolution of ~120 nm (super-resolution, achieved with a 488 nm laser), spatial structured illumination microscopy (3D-SIM)

was applied using a Plan-Apochromat 63x/1.4 oil objective of an Elyra PS.1 microscope system and the software ZENblack (Carl Zeiss GmbH)[58].

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Raw Illumina short read sequencing data were deposited under BioProject PRJNA602906. TBP genotyping data can be found here: https://figshare.com/articles/dataset/Lemna_minor_Tubulin-based_polymorphism_TBP_amplified_fragments/29609492?file=56422559. All other data are available from the corresponding authors on reasonable request.

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Author contributions

T.P.M., E.L. and B.W.A. conceived the project. Y.F.C., B.P., and E.L. maintained the *L. minor* collection at the RDSC. K.C., T.D. and M.L. grew, sterilized, and extracted DNA from *L. minor* clones. S.P., N.H., A.M. and N.A. processed short read sequencing data, estimated genome size, assembled short reads, mapped short reads, estimated variation and constructed pangenome. P.T.N.H., V.S., and I.S. provided chromosome counts and GISH. J.F. and M.W. estimated genome size using flow cytometry. L.B. and L.M. validated species designation using the Tubulin gene intron-Based Polymorphism (TPB) molecular markers. E.E., T.P.M., and R.A.M. generated reference assemblies and annotations for *L. gibba*, *L. x japonica* and *L. turionifera* clones before publication. K.S.S., K.J.A., M.B., B.P. and E.L. conducted growth rate experiments and supported interpretation of genome size data. M.B. measured frond area. T.P.M. and E.L. wrote the initial manuscript and the other co-authors revised and edited.

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Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Todd P. Michael, Robert A. Martienssen or Eric Lam.

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[1]The Plant Molecular and Cellular Laboratory, Salk Institute for Biological Studies, La Jolla, CA, USA. [2]Department of Cell and Developmental Biology, School of Biological Sciences, and Center for Marine Biotechnology and Biomedicine, University of California, San Diego, CA, USA. [3]Department of Science and Conservation, San Diego Botanical Garden, Encinitas, CA, USA. [4]Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Seeland, Germany. [5]Biology Faculty, Dalat University, Lamdong Province, Vietnam. [6]Department of Plant Biology, Rutgers the State University of New Jersey, New Brunswick, NJ, USA. [7]Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA. [8]Institute of Agricultural Biology and Biotechnology, National Research Council (CNR), Milan, Italy. [9]Rutgers Duckweed Stock Cooperative, New Brunswick, NJ, USA. [10]Institute of Botany and Landscape Ecology, University of Greifswald, Greifswald, Germany. [11]School of Biotechnology, Institute of Science, Banaras Hindu University, Varanasi, India. [12]Matthias Schleiden Institute-Genetics I, University of Jena, Jena, Germany. [13]Integrative Genetics and Genomics Graduate Group, Plant Sciences Department, University of California Davis, Davis, CA, USA. [14]Present address: Noblis Inc., Washington, DC, USA. [15]Present address: Cepheid, Sunnyvale, CA, USA. ✉e-mail: toddpmichael@gmail.com; tmichael@salk.edu; martiens@cshl.edu; eric.lam@rutgers.edu