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BRIEF COMMUNICATION

Newly unveiled meiosis elucidates the unreduced gamete frequency and its impact on the evolution of the *Lemna minor* complex

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Abstract

Fusion of gametes possessing meiotically reduced (haploid) chromosome complements is the main pathway of propagation among eukaryotes. However, duckweeds, the smallest angiosperms, propagate mainly vegetatively, and meiosis has not yet been documented in detail for this plant family. More surprising was the recent evidence of rather frequent interspecific hybrids and triploid clonal accessions, which became obvious by genome size measurements, genomic *in situ* hybridization (GISH), and combined plastid and nuclear DNA markers. These observations indicated sexual propagation involving reduced as well as unreduced male and female gametes in *Lemna minor* and *L. turionifera*, leading to allodiploid and allotriploid hybrids (with genome compositions MT, MMT, MTT) and autotriploid *L. minor* (MMM) accessions. Here, we (i) document the meiotic stages of *Lemna* species for the first time photographically; (ii) provide evidence of unreduced male gametes through fluorescent *in situ* hybridization (FISH) with single locus probes; (iii) determine their abundance in different individuals; and (iv) discuss possible reasons for unreduced male gamete formation. These findings open new insights into the modes of sexual reproduction and evolution of duckweeds, which may be useful for future breeding efforts in this emerging crop.

Keywords: Duckweed, duckweed flower, interspecific hybrids, *Lemna* section *Lemna*, meiosis, sexual reproduction, unreduced male gametes.

Introduction

Meiosis, as a reductive cell division, is essential for sexual reproduction, including recombination of paternal and maternal alleles. It comprises a single round of DNA replication followed by two cell divisions, separating homologous chromosomes during the first, and sister chromatids during the second division, thus generating cells with half the number of

chromosomes of the somatic mother cell. However, meiotic defects can produce viable gametes retaining somatic chromosome numbers, potentially leading to polyploid progeny (Brownfield and Köhler, 2011; Zamariola *et al.*, 2014). Plant polyploidy is widespread in natural ecosystems and in many major crops (Brownfield and Köhler, 2011). Therefore, understanding the cytological events underlying normal meiosis as well as the formation of unreduced gametes is fundamental for elucidation of evolutionary processes, and provides a basis for plant breeding.

Duckweeds, the smallest flowering angiosperms, are an aquatic monocot family (Lemnaceae Martinov) which mainly reproduce through fast asexual propagation. *Lemna* L., the largest of five duckweed genera, comprises four sections (*Alatae* Hegelm., *Biformes* Landolt, *Lemna* L., and *Uninerves* Hegelm.), including so far 11 species and three interspecific hybrids, as well as ploidy variants and backcrosses (for review see Morello *et al.*, 2026). Even though duckweeds have been studied for over two centuries, due to their small size (0.6–9 mm in diameter), simple organismic structure (leaf-like fronds with or without roots), and rare flowering under natural and/or experimental conditions, their taxonomy and phylogeny remained unclear until recently.

Recent applications of advanced molecular and cytological approaches have revealed surprisingly frequent auto- and allopolyploids, which require sexual propagation (Braglia *et al.*, 2024; Ernst *et al.*, 2025; Michael *et al.*, 2026; Stepanenko *et al.*, 2025). Notably, the representative interspecific hybrid in sect. *Lemna*, *Lemna* × *japonica* Landolt, results from unidirectional crosses between *L. minor* L. (M) ♀ and *L. turionifera* Landolt (T) ♂, and exhibits variation in ploidy and genome composition (MT, MMT, MTT) (Braglia *et al.*, 2021; Ernst *et al.*, 2025), implying recurrent hybridization and the occurrence of unreduced gametes in both parental species. Consistent with this inference, naturally occurring autotriploid *L. minor* accessions have also been reported (Michael *et al.*, 2026). However, direct experimental confirmation of unreduced gamete formation has been lacking so far.

In most flowering plants, interploidy crosses via fusion of reduced and unreduced gametes usually result in abnormal endosperm development and seed abortion, called 'triploid block' (Cooper and Brink, 1945; Marks, 1966; Köhler *et al.*, 2021). Duckweed can overcome this reproductive barrier, at least in part, possibly through the loss of genes of RNA-dependent DNA methylation (RdDM) pathways (Ernst *et al.*, 2025). Their predominant clonal growth facilitates the persistence of triploid lineages and their sympatric diversification, despite sterility (Lee *et al.*, 2026). Consequently, knowledge about the frequency of reduced and unreduced gametes in duckweeds is instrumental for elucidating their evolutionary dynamics.

Due to many small chromosomes and only a few meristematic cells, reliable cytological studies are scarce for duckweeds (e.g. Cao *et al.*, 2016; Hoang and Schubert, 2017; Hoang *et al.*, 2019, 2020, 2022; Stepanenko *et al.*, 2025). Moreover, the rarity of natural flowering, the difficulty of inducing flower formation under experimental conditions, and the smallness of flowers (flower <1 mm with one pistil and asynchronously growing two stamens surrounded by a spathe; see Supplementary Fig. S1 in Lee *et al.*, 2026) prevented detailed studies of meiosis, despite the obvious need for assessing the evolutionary prospects of duckweeds. We are aware of only a very limited number of studies mentioning duckweed meiosis (Caldwell, 1899; Maheshwari and Kapil, 1963) without photo-documentation.

This study provides the first detailed cytological characterization of male meiotic stages, a protocol for their preparation from young flowers, and for analysing the diverse final meiotic products in the duckweed section *Lemna*. By examining *L. minor* and *L. turionifera*, we quantified the meiotic products of different ploidy levels, and assessed the occurrence and frequency of reduced and unreduced gametes in these predominantly clonally propagating plants.

Materials and methods

Plant materials and culture conditions

All tested duckweed accessions were originally from the Landolt collection and are now available at the duckweed collection of the Institute of Agricultural Biology and Biotechnology (CNR-IBBA) in Milan (Italy; Morello *et al.*, 2025). The diploid accessions *L. minor* 5500, *L. turionifera* 9434 and 7432 were selected based on prior investigations of their floral development, as well as their genetic and cytogenetic features (Braglia *et al.*, 2021; Ernst *et al.*, 2025; Lee *et al.*, 2026). Plants were maintained under axenic conditions (see Braglia *et al.*, 2021).

Preparation of meiotic cells

To prepare meiotic stages, young developing anthers were collected from artificially induced flowers (Lee *et al.*, 2026). In brief, few fronds were pre-cultured for seven days in 50 ml plastic beakers with 20 ml of SH medium (Schenk and Hildebrandt, 1969), including 1% sucrose. For flower induction (FI), some asexually propagating, healthy fronds were then placed in 100 ml plastic beakers with 100 ml of modified NH_4^+-free 5% Hutner liquid medium (Lee *et al.*, 2026) and 30 μM salicylic acid (SA). The plants were kept under a 16 h light/8 h dark photoperiod (white light, 150 μmol m^{-2} s^{-1}) at 25 °C. Considering that *Lemna* plants usually start flowering from days 12–14 of FI (Lee *et al.*, 2026), at day 9, fronds were transferred into 90×20 mm petri dishes containing semisolid FI medium (with 0.3% agar), to monitor for expected immature flowers (possibly undergoing meiosis).

Fronds bearing young flowers were confirmed by checking the underside of the petri dish under a stereo microscope (SMA18, Nikon, Tokyo, Japan) (Fig. 1). Immature flowers of different sizes were independently dissected and immediately fixed in a freshly prepared 3:1 (v:v) ethanol: acetic acid solution for 3–6 d at 4 °C, then transferred to 70% ethanol and stored at −20 °C until further use.

Prior to slide preparation, fixed flowers were treated with 45% acetic acid for up to 30 min. Single anthers were transferred onto glass slides with 1 μl of 45% acetic acid, and further dissected using hypodermic needles (18G) to release the meiotic cells. After adding 7 μl of 45% acetic acid, the cell suspension was covered with a coverslip. Slides were briefly flamed over a burning ethanol burner (~1 s), then immersed in liquid nitrogen for 10 s. The coverslip was subsequently removed using a razor

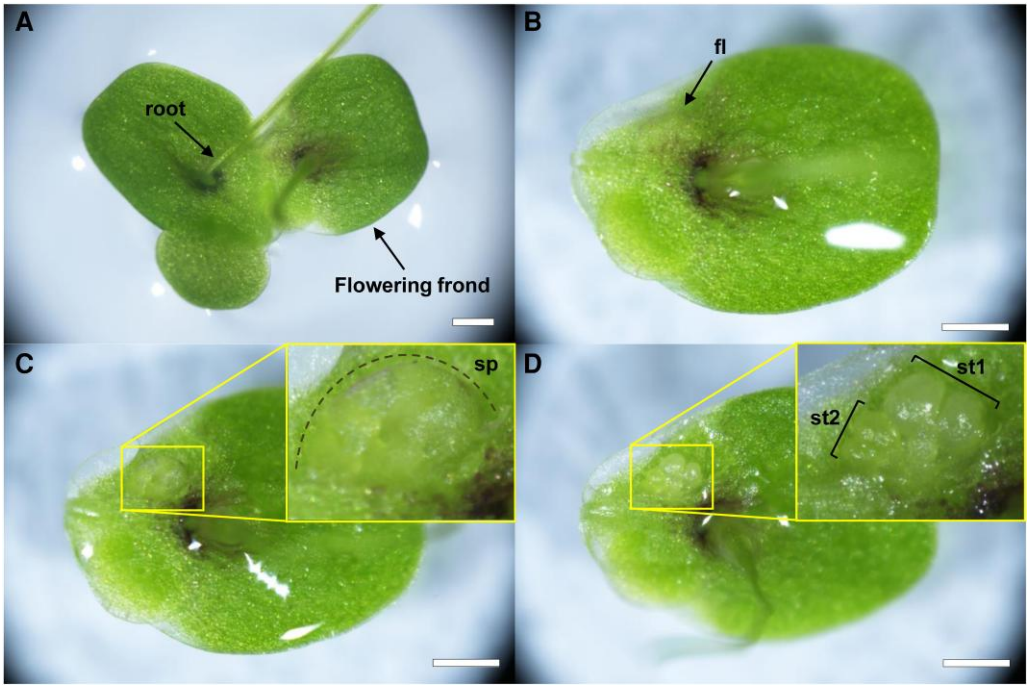

Fig. 1. Different preparation stages of one individual of *Lemna turionifera* clone 7432 bearing immature flower. (A) Abaxial side of three cohering fronds, one of which is flowering; (B) the detached flowering (fl) frond; (C) the young flower surrounded by the reddish spathe (sp) is visible after removing frond's abaxial surface; (D) the young, developing primary stamen (st1) and the secondary stamen (st2) are visible after removing the spathe. Scale bars=500μm.

blade, and the slides were immediately placed in pre-chilled 99.8% ethanol at −20 °C.

Probe generation and fluorescence *in situ* hybridization (FISH)

Lemna-specific 5S and 45S rDNA probes were generated following the procedure described by Hoang and Schubert (2017) and Stepanenko *et al.* (2026) with small modifications. Total DNA was extracted from plant tissue using a modified CTAB method (Murray and Thompson, 1980). First, genomic DNA isolated from *L. minor* (clone 8703) was amplified using duckweed rDNA-specific primers (Hoang and Schubert, 2017; Stepanenko *et al.*, 2026). After purification, the obtained PCR products were used as templates for labeling with specific fluorescent dyes. The rDNA fragments were subjected to nick-translation using the AZDye594 NT Labeling Kit for 45S rDNA (red) and the AZDye488 NT Labeling Kit for 5S rDNA (green) (Jena Bioscience, Jena, Germany). After precipitation with 96% ethanol, probe pellets were dissolved in 100 μl hybridization buffer (50% (v/v) formamide, 20% (w/v) dextran sulfate in 2×SSC, pH 7).

The meiotic products prepared on slides were denatured in 2N NaOH for 5 min at room temperature (22~25 °C), rinsed for stepwise dehydration in 75% (twice) and 99.8% ethanol for 5 min each, and air-dried. Of each FISH probe, 1 μl (50 ng μl^{-1}) was added to 10 μl hybridization mixture [dextran sulphate (1 g) dissolved in 1 ml ddH_2O, followed by addition of 5 ml deionized formamide, 1 ml 20×SSC, and 1 ml sonicated fish sperm DNA], denatured at 99 °C for 15 min, and stored at −20 °C until use. The mixture was applied to air-dried slides, sealed with coverslips, and incubated in a moist chamber at 37 °C overnight (Aliyeva-Schnorr *et al.*, 2015). After hybridization, the slides were rinsed twice in 2×SSC for 5 min, followed by distilled water for 2 min, and then air-dried. Finally, 10 μl of 4′,6-diamidino-2-phenylindole (DAPI) antifade solution (1 μg ml^{-1}) was added to each slide and sealed with a coverslip.

Microscopy

To detect the ultrastructural chromatin organization of cells at a resolution of ~120 nm (super-resolution achieved with a 488 nm laser excitation), spatial structured illumination microscopy (3D-SIM) was performed with a 63×/1.4 Oil Plan-Apochromat objective of an Elyra 7 microscope system and the software ZENBlack (Carl Zeiss GmbH, Jena, Germany). Images were captured separately for each fluorochrome using the 405, 488, and 561 nm laser lines for excitation and appropriate emission filters (Weisshart *et al.*, 2016). The spatial animation was done based on the SIM image stack (Supplementary Video S1) using Imaris 9.7 (Bitplane, USA) software. The tool 'Surface' was applied to indicate nuclei volumes. Microspores stained with DAPI were counted using the fluorescence microscope AXIO Lab.A1 (Carl Zeiss GmbH). In total, 17 anthers from 17 individual ramets representing the three clones were individually mounted on slides and investigated.

Results

Microscopic observation and determination of the male meiotic stages

Pollen mother cells at the zygotene stage in *L. turionifera* 9434 and *L. minor* 5500, and at the pachytene stage in *L. turionifera* 7432, were observed when anthers just became detectable at sizes of 143×134 μm, 157×119 μm, and 131×91 μm; length×width, respectively. Pollen mother cells of both *Lemna* species stained with DAPI displayed the typical meiotic stages, such as zygotene, pachytene, metaphase I and II, similar to those of other eukaryotes (Fig. 2). Metaphase I of *L. turionifera* 9434 and 7432 showed the presence of 21 bivalents, indicating a haploid

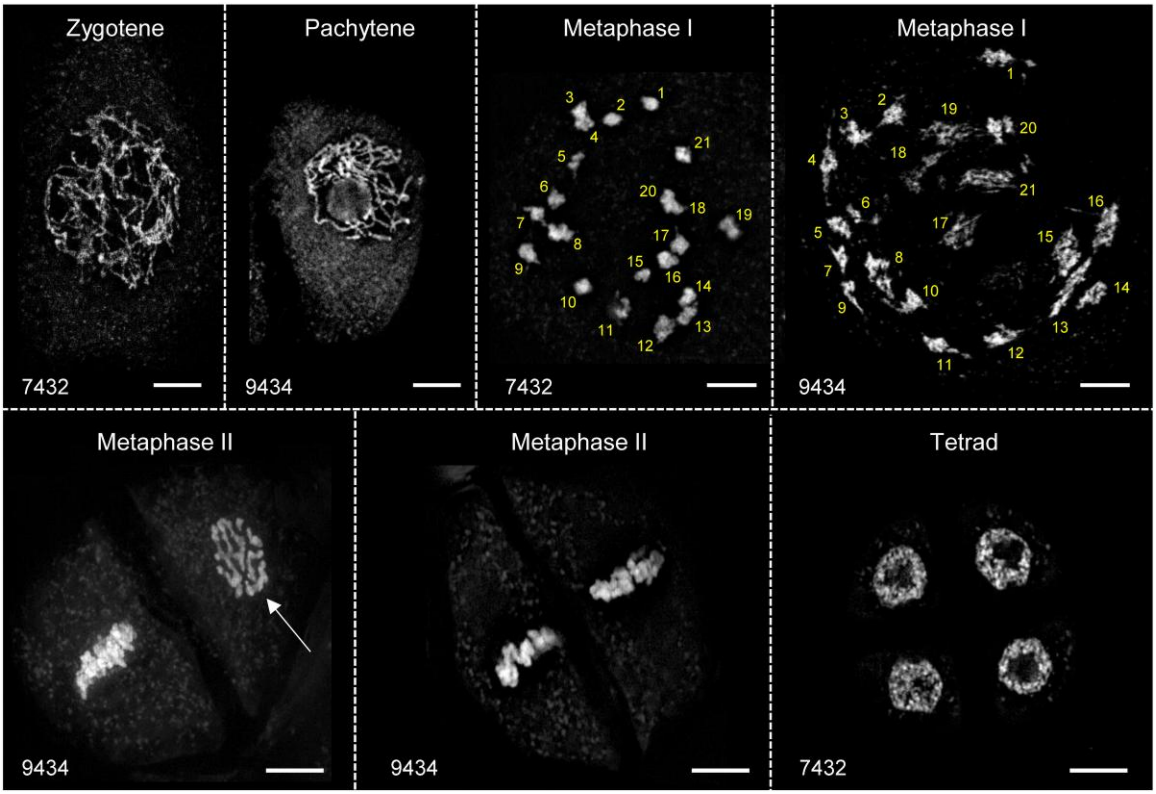

Fig. 2. Meiotic stages of *Lemna turionifera* (accessions 9434 and 7432). Zygotene shows that homologous chromosomes start pairing. Chromosome pairing is complete in pachytene. Metaphase I contains 21 bivalents (numbered) in both accessions. Two photos of metaphase II show the chromosome arrangement from different orientations. Scale bars=5μm.

chromosome number relative to the somatic complement. Meiosis was regular in both species, and the distinct meiotic stages appeared synchronously.

Tetrads as the meiotic end-product were detected in developing anthers with mean (±SD) sizes of (175±15)×(144±20) μm for *L. turionifera* 9434 (n=4), (196±12)×(153±8) μm for *L. turionifera* 7432 (n=7), and (219±14)×(181±12) μm for *L. minor* 5500 (n=6). The anther sizes corresponding to the tetrad stage seem variable amongst the three accessions, suggesting that anther size alone cannot serve as an absolute criterion for identifying the developmental stage, even within the same species (*L. turionifera*). At the tetrad stage of the primary anther, the secondary anther typically exhibited cells at the zygotene to metaphase I stages.

Detection of various meiotic products and confirmation of the number of nuclei in each cell

At the last stage of meiosis, across all three accessions, most tetrads displayed a tetragonal-like arrangement of the resulting four cells. Flat clover leaf- and T-shaped forms were also observed, albeit at a lower frequency. In addition to tetrads, dyads and triads were also detected. In total, 4710 cells were observed. *Lemna turionifera* 9434 and 7432 produced 96.9% and 98.8% reduced gametes as well as 3.1% and 1.2% unreduced gametes, respectively. *Lemna minor* 5500 showed 96.3% reduced and 3.7% unreduced gametes (Table 1). The frequency of different meiotic end-products varied not only between accessions but also among individuals within the same accession. Cells of dyads and their nuclei were noticeably larger, about double the size than those of tetrads (Supplementary Fig. S1 and Videos S1, S2). Thus, tetrad formation is not due to larger meiocytes, compared with those resulting in unreduced dyads, as observed for *jason* (*jas*) mutants in Arabidopsis (Yi *et al.*, 2023).

FISH revealed the same number of rDNA signals in dyads within their two (or already fused) nuclei, as observed in somatic cell nuclei. However, each tetrad cell nucleus showed only one 45S rDNA signal (Fig. 3). This was to be expected for single loci of 5S and 45S rDNA, as reported for *Lemna* species so far (Hoang *et al.*, 2019). Therefore, the presence of tetrads, dyads, and a few triads is consistent with the occurrence of reduced and unreduced gametes in the three accessions of the two frequently hybridizing *Lemna* species.

Discussion

Unreduced gametes, as the most important source of polyploids (followed by early somatic chromosome doubling of

Table 1. Numbers and ratios of reduced (1n) and unreduced (2n) gametes derived from diverse meiotic products (dyads, triads, and tetrads) in individual anthers of three *Lemna* accessions

	2n gamete number from dyad	2n gamete number from triad	1n gamete number from triad	1n gamete number from tetrad	2n gamete rate (%)	1n gamete rate (%)
Lemna turionifera 9434	40	0	0	544	6.85	93.15
	2	0	0	440	0.45	99.55
	12	2	4	348	3.83	96.17
	0	0	0	404	0.00	100.00
total	54	2	4	1736	3.12	96.88
L. turionifera 7432	2	2	4	420	0.93	99.07
	4	0	0	556	0.71	99.29
	0	0	0	112	0.00	100.00
	4	0	0	580	0.68	99.32
	0	0	0	412	0.00	100.00
	4	2	4	404	1.45	98.55
	6	4	8	188	4.85	95.15
total	14	6	12	1584	1.24	98.76
L. minor 5500	2	0	0	372	0.53	99.47
	4	1	2	200	2.42	97.58
	2	2	4	268	1.45	98.55
	32	11	22	292	12.04	87.96
	6	0	0	336	1.75	98.25
	0	0	0	452	0.00	100.00
total	40	13	26	1348	3.71	96.29

diploid zygotes), can arise through multiple cytological pathways (Brownfield and Köhler, 2011). In plants, defective synapsis, abnormal spindle orientation, omission of meiosis I and/or II, impaired cytokinesis, and restitution of nuclei were reported as causes for unreduced gametes (Peloquin *et al.*, 1989; Bretagnolle and Thompson, 1995; De Storme and Geelen, 2011). Consistent with these mechanisms, our observations revealed restitution of nuclei after lacking cytokinesis during male meiosis II in *L. minor* and *L. turionifera*. These findings are the first direct evidence showing the proportions of defective products of meiosis for two *Lemna* species (Table 1, Fig. 3).

Studies in *Arabidopsis thaliana* (L.) Heynh. have identified several core meiotic regulators, such as *Arabidopsis thaliana* Parallel Spindle 1 (AtPS1), JASON (JAS), Omission of Second Division 1 (OSD1), Cyclin A (CYCA1;2/TAM), SUMO E3 ligase (AtMMS21) and apparently also Structural Maintenance of Chromosome 5/6 (SMC5/6), which, when disrupted, deleted or down-regulated, may cause meiotic defects and result in high frequencies of unreduced gametes (d'Erfurth *et al.*, 2008, 2010; De Storme and Geelen, 2011; Liu *et al.*, 2014; Yang *et al.*, 2021). Based on genomic sequences, Ernst *et al.* (2025) proposed that *L. turionifera* and *L. minor* accessions might exhibit elevated unreduced male gamete production due to deletions in JASON homologs, which are responsible for forming organelle bands and correct cytokinesis during male meiosis II (Brownfield *et al.*, 2015). Our cytological data revealed marked inter-individual variation within this accession (*L. turionifera* 9434), with unreduced gamete frequencies ranging from 0 to 7% between individuals. Because this is substantially lower than reported for *jas* mutants of *A. thaliana* (De Storme and Geelen, 2011), a mutation of JASON as an explanation for the observed variation in *Lemna* could at best be of low penetrance. *OSD* and *TAM* mutations are less likely to be the reason for the observed unreduced meiotic products, because fusion of dyad nuclei indicates that at least division of nuclei during meiosis II was not omitted. Mutations of SMC5/6 components could also mediate formation of unreduced gametes (Yang *et al.*, 2021), albeit it remains unclear how such mutations disturb meiosis II preferentially, because SMC5/6 is mainly required for meiosis I.

In wild-type angiosperms lacking obvious mutations in core meiotic regulators, spontaneous unreduced gamete formation is generally rare, typically below 2% in natural populations (Ramsey, 2007; Kreiner *et al.*, 2017). The *Lemna* individuals examined here showed pronounced variation in unreduced gamete frequencies among individuals of the same clonal accessions, between 0–12% (Table 1). While most individuals produced unreduced gametes at lower frequencies (~1–5%), a single flower of *L. minor* yielded 12% (27% of male meiotic end-stages were dyads and triads). Given the rapid clonal propagation characteristic of *Lemna*, it is possible that somatic mutations affecting meiotic stability were acquired independently among and within different clonal accessions, contributing to this heterogeneity.

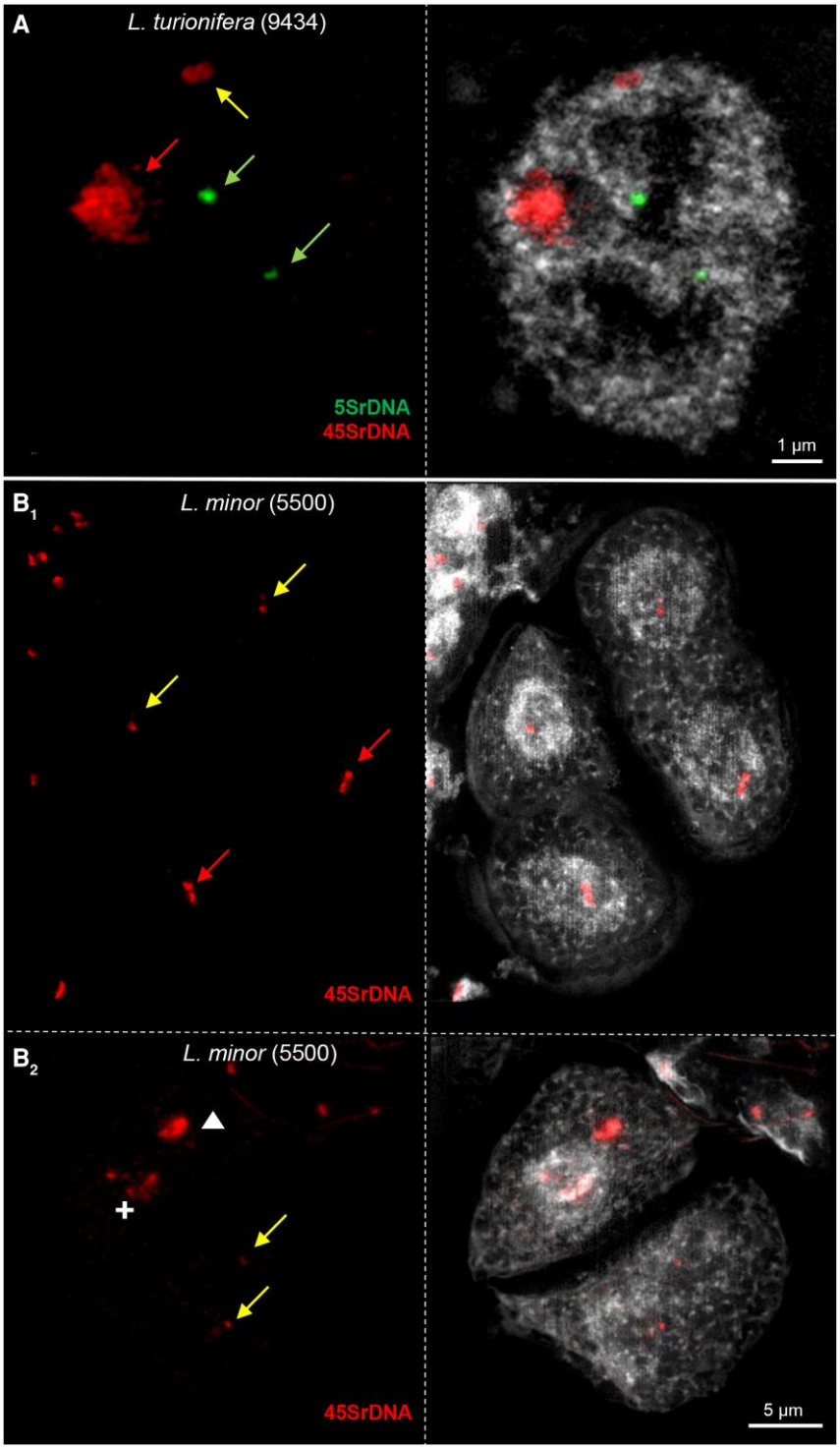

Fig. 3. Fluorescence *in situ* hybridization (FISH) with rDNA detects unreduced gametes in the genus *Lemna*. (A) A single slice of a 3D-SIM image stack of a somatic interphase nucleus of *L. turionifera* accession 9434 showing an extended major 45S rDNA signal attached to a nucleolus (red arrow) and a minor signal at the edge of another nucleolus (yellow arrow). Both homologous 5S rDNA signals are marked by green arrows. (B1) Minor 45S rDNA signals (yellow arrows) are visible in the two nuclei of the top cells of a tetrad. Major signals (red arrows) are in the bottom cells. [Copy number heterozygosity between homologous rDNA loci was also documented for *Spirodela polyrhiza* 5S rDNA by Stepanenko *et al*. (2026)]. (B2) One of the major 45S rDNA signals in an unreduced dyad is dispersed within the nucleolus of the top cell (cross), the second homologous locus appears condensed outside (triangle). The bottom cell contains the two minor signals of the second homolog (yellow arrows). The small cells adjacent to the dyad are part of the tapetum cells. Both images in (B) are maximum intensity projections (see also Supplementary Videos S1 and S2).

As an alternative to mutation of meiotic genes, environmental stress causing a switch to sexual propagation, including emergence of a few unreduced gametes, could be responsible for natural auto- and allopolyploids. *In vitro* flowering requires hormonal treatments that mimic natural stress responses, and such stress has been linked to increased frequencies of unreduced gametes in plants (Ramsey and Schemske, 1998). Under unfavorable conditions that constrain asexual propagation, sexual reproduction could therefore contribute to adaptive genetic diversity, potentially through polyploidization and hybrid formation (Mason and Pires, 2015).

Although not easily accessible, unreduced *L. minor* female gametes should also occur, since triploid *L.* × *japonica* with two genomes of *L. minor* were found, albeit crossing between *L. minor* × *L. turionifera* was exclusively unidirectional as was shown by plastid markers (Braglia *et al.*, 2021; Ernst *et al.*, 2025).

Despite their predominantly clonal reproduction, *L. minor* and *L. turionifera* conform to general patterns reported across angiosperms (Bretagnolle, 2001; Ramsey, 2007), in which a higher frequency of unreduced gametes is restricted to relatively few individuals. Notably, tetraploid *L.* × *japonica* has not been reported so far. Low flowering rates of the parental species in natural populations (1.5–5%; Landolt, 1986, pp. 167–169), together with low frequencies of unreduced gametes, substantially reduce the probability of fusion of unreduced gametes from both parents. This explanation may also account for the absence of autotetraploid *L. minor* and *L. turionifera*.

Recent evidence from *L.* × *aoukikusa* Beppu et Murata suggested that at least some *Lemna* taxa rapidly absorb the endosperm during embryo development (Lee *et al.*, 2026), indicating that the triploid block may not be of major impact in genus *Lemna*. It is possible that the dependence on stored reserves provided by the endosperm may be reduced for duckweeds, due to their aquatic lifestyle, which enables photosynthesis immediately after germination of seedlings.

Our study provides the basis for comparative cytological analyses of meiotic stages in duckweeds (Figs 1, 2). Diverse natural allopolyploids are even more common in sect. *Alatae* (Stepanenko *et al.*, 2025) than in sect. *Lemna*, exemplified by the fertile tetraploid taxon *L.* × *aoukikusa* and its triploid backcrosses with *L. aequinoctialis* Welw. It is most likely that interspecific hybrids also occur in the genus *Wolffia* Horkel ex Schleid. Thus, knowledge on how to address meiotic processes (this study) as well as species fertility and crossability (Lee *et al.*, 2026) will uncover lineage-specific routes underlying polyploidization and diversification in duckweeds, and provide prerequisites to harness crossing, and thus domestication of economically interesting duckweeds.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. Dyad (top) and tetrad of *Lemna turionifera* (9434).

Video S1. 3D animation of the *Lemna minor* (5500) dyad shown in Fig. 3B2. The green spheres indicate the nuclei in both cells before fusion into restitution nuclei.

Video S2. Image stack of the *Lemna minor* (5500) dyad shown in Fig. 3B2.

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Author contributions

YL, IS, and LM: Conceptualization; YL, VS, AS, GK, and LB: Investigation & Methodology; YL, VS: Visualization; YL: Writing—Original Draft Preparation; IS, LM: Supervision & Writing—Review & Editing; LM and LB: Project Administration & Resources.

Conflict of interest

No conflict of interest declared.

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Data availability

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

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