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RESEARCH PAPER

Characterization of the cryptic interspecific hybrid *Lemna×mediterranea* by an integrated approach provides new insights into duckweed diversity

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Abstract

Lemnaceae taxonomy is challenged by the particular morphology of these tiny free-floating angiosperms. Although molecular taxonomy has helped clarify the phylogenetic history of this family, some inconsistency with morphological data leads to frequent misclassifications in the genus *Lemna*. Recently, the finding that *Lemna japonica* is an interspecific hybrid between *Lemna minor* and *Lemna turionifera* provided a clear explanation for one such taxonomic question. Here we demonstrated that *L. minor* is also capable of hybridizing with *Lemna gibba*, generating a cryptic but widespread taxon in the Mediterranean area. The nothotaxon *Lemna ×mediterranea* is described and compared with clones of the putative parental species *L. minor* and *L. gibba*. Genetic analysis by nuclear and plastid markers, as well as genome size measurement, revealed that two different cytotypes, diploid and triploid, originated by at least two independent hybridization events. Despite high overall similarity, morphometrical, physiological, and biochemical analyses showed an intermediate position of *L. ×mediterranea* between its parental species in most qualitative and quantitative characters, and also separation of the two hybrid cytotypes by some criteria. These data provide evidence that hybridization and polyploidization, driving forces of terrestrial plant evolution, contribute to duckweed genetic diversity and may have shaped the phylogenetic history of these mainly asexual, aquatic plants.

Keywords: Aquatic plants, cytotype, DNA barcoding, duckweed, interspecific hybrids, *Lemna gibba*, *Lemna mediterranea*, *Lemna minor*, morphometry.

Introduction

The Lemnaceae family is exclusively composed of aquatic plants (commonly named duckweeds) that are the smallest flowering plants, showing a body plan reduced to a single leaf-like structure called a frond, without or with one or a few roots. The main morphological traits are limited to frond shape, size and colour, root number and length, and position and number of vegetative pouches (Landolt, 1986). Additional diagnostic traits are vein number, and the presence of a prophyllum at the base of the root(s) or papules on the dorsal side of the frond. Flowers, fruits, and seeds provide important additional taxonomic traits but are rarely or never observed in some species, as duckweeds mostly propagate asexually by forming daughter fronds from vegetative pouches on the mother frond. Key morphological features for each species were recently updated (Bog *et al.*, 2020a), but classification by morphology in some cases remains insufficient as not all specimens are assignable to one of the 36 recognized species with confidence. A detailed morphometric analysis has proven helpful to distinguish the American species *Lemna minuta* Kunt, invasive in Europe, from the native *Lemna minor* L. (Ceschin *et al.*, 2016). The problem has been partially overcome with the introduction of molecular taxonomy that provided new instruments for species delimitation. Barcoding plastid markers (Les *et al.*, 2002; Wang *et al.*, 2010; Borisjuk *et al.*, 2015) and nuclear sequences, such as internal transcribed spacers (ITSs), external transcribed spacers (ETSs) (Tippery *et al.*, 2015), as well as amplified fragment length polymorphism (AFLP) (Bog *et al.*, 2015, 2019), mostly contributed to an almost complete phylogenetic reconstruction of the Lemnaceae family, which includes five monophyletic genera: *Lemna*, *Spirodela*, *Landoltia*, *Wolffia*, and *Wolffiella* (Les and Crawford, 1999). Nevertheless, some species remain poorly delimited, particularly in the genera *Wolffia* and *Wolffiella* (Tippery *et al.*, 2015; Bog *et al.*, 2019). In the genus *Lemna*, inconsistency between nuclear and plastid markers impairs taking apart clones of *Lemna japonica* Landolt (Landolt, 1980), often mistaken for *Lemna minor*: their plastid barcoding sequences are in fact almost identical. This issue was recently solved by using tubulin-based polymorphism (TBP), a nuclear molecular marker based on the intron length polymorphisms of the β-tubulin gene family members, which provided evidence that this species is an interspecific hybrid between *L. minor* and *Lemna turionifera* (Braglia *et al.*, 2021a). This was recently confirmed by whole-genome sequencing of three different *Lemna ×japonica* clones flanked by genomic *in situ* hybridization (GISH) analysis (Ernst *et al.*, 2023, Preprint) revealing that hybrids can form as both diploids and reciprocal triploids.

Lemna minor also shares many morphological traits with the sister species *Lemna gibba* L., and distinction of the two may be challenging in some cases. Usually, *L. gibba* specimens are easily identified for the pronounced gibbosity of the ventral side of its fronds, due to a diffused and inflated aerenchyma, but this trait is partially influenced by growth conditions that in some cases do not make it as noticeable (Landolt, 1986). In addition, intermediate forms that cannot be determined with certainty have been reported in the Netherlands (De Lange and Pieterse, 1973; Kandeler, 1975; Landolt, 1975; De Lange and Westinga, 1979) so that the two species have been described as forming a species complex (De Lange *et al.*, 1981). Interestingly, a new species similar to *L. gibba* was described in Italy in 1973 under the name *L. symmeter* Giuga (Giuga, 1973). However, the description of this species was not validly published following the criteria of the time (no Latin description), and was almost forgotten. *Lemna symmeter* had been identified at several sites along the coast of the Campania region (Southern Italy) and described as similar to the strongly globose *L. gibba*, but only slightly ventricose and with smaller aerenchyma spaces. In particular, the two species were described as easily distinguished for the symmetric growth of the two stamens in *L. symmeter*, compared with the asynchronous growth in *L. gibba*. While *L. gibba* was reported to produce fruits and seeds, *L. symmeter* was described as sterile, producing abortive ovules and indehiscent anthers (Giuga, 1973). Kandeler (1975) hypothesized that *L. symmeter* could be an interspecific hybrid between *L. gibba* and *L. minor*, as also later reported by Landolt (1986), but this possibility was never investigated thereafter.

More recently, non-gibbous forms of *L. gibba*-like specimens of uncertain taxonomic assignment were described at some sites in Central Italy (Marconi *et al.*, 2019). However, when analysed by plastid markers, these specimens were all assigned to *L. minor*, supporting the idea of morphological variants of this species. One of the clones isolated during that study was sent to the Landolt collection and registered as 9562; it is analysed here and designated as the hybrid type.

The existence of natural interspecific hybrids between *L. minor* and *L. gibba* was finally hypothesized, upon a large screening of clones belonging to the *Lemna* genus present in the Landolt Duckweed Collection (Braglia *et al.*, 2021b). Similar to *L. ×japonica*, the new hybrid taxon was first identified on a molecular basis by TBP fingerprinting and reported with the hybrid formula *L. gibba* × *L. minor*. This finding accounts for the erroneous species assignment using plastid markers of maternal origin.

The main aims of this study are: (i) to fully demonstrate on a genetic basis the hybrid nature of the six clones previously identified, plus an additional one (LM0027) successively recovered from the Botanical Garden of Naples (Italy), and (ii) to characterize this interspecific *Lemna* hybrid based on morphological, physiological, and biochemical traits in comparison with clones of the two parental species. Such characterization is supported by molecular analysis of plastid and nuclear markers of the six original clones of the Landolt Collection plus an additional clone from the Botanical Garden of Naples (Italy).

Materials and methods

Plant material

Seven putative hybrid clones, here assigned to the hybrid taxon *L.* ×*mediterranea*, were analysed in comparison with several clones of the two parental species, *L. minor* and *L. gibba*, by different approaches. Most analysed clones originated from the historical living plant collection of Professor Elias Landolt (Lämmler and Bogner, 2014), presently maintained as part of the IBBA collection (Milano, Italy), while others came from other collections in Europe or were collected in Italy by the authors and integrated into the IBBA collection. All clones are listed in Table 1 with the name of the donor, collection site and date, and the experiment in which they have been used.

Propagation of duckweed clones

Axenic stock cultures were maintained in Petri dishes on agarized Schenk and Hildebrandt (SH) medium, pH 5.1 (plus 8 g l^{-1} Plant Agar, Duchefa) supplemented with 0.1% sucrose, under the following growth conditions: temperature, 18 °C; photoperiod, 16 h day, 8 h night; light intensity, 80 ± 10 µmol m^{-2} s^{-1}. For each set of analysis/measurements, plants were transferred into liquid medium or water, as described in the specific experimental section.

DNA extraction and quantification

DNA extraction was performed from ~100 mg FW, using the DNeasy Plant Mini Kit (QIAGEN) as reported previously (Braglia *et al.*, 2021a) and eluted in 150 µl of 50 mM Tris, pH 9. When necessary, DNA was more precisely quantified through the dsDNA HS Assay Kit for the Qubit fluorometer (Thermo Fisher Scientific).

Relative genome size measurement

To investigate hybrid ploidy, relative genome size measurements were performed using a CyFlow Space flow cytometer (Sysmex Partec GmbH, Görlitz, Germany). To extract nuclei from fresh plant tissue, ~3–4 fully grown fronds of the internal standard *Lemna aequinoctialis* Welw. (6746) and 2–3 fully grown fronds of the sample were chopped carefully in 500 µl of Otto I buffer [0.1 M citric acid, 0.5% (v/v) Tween-20; Ulrich and Ulrich, 1991] with a sharp razor blade. The extract was incubated for 5 min on ice and then filtered (~30 µm filter size). Subsequently, 500 µl of the staining Otto II buffer (0.4 M Na_2HPO_4, 4 mg ml^{-1} DAPI; Ulrich and Ulrich, 1991) were added and the sample was measured after incubation for 5 min in darkness in the flow cytometer equipped with a 375 nm UV laser. Data collection was stopped after a minimum of 10 000 events, and the relative genome sizes were calculated as the proportion of fluorescent intensities of the sample relative to the internal standard. Seven samples were measured in replicates, but did not show differences in their sample:standard ratios.

TBP amplification

TBP amplification, amplicon separation by capillary electrophoresis (CE), and fragment analysis were performed as reported in Braglia *et al.* (2023) with minor variations. Amplification of specific β-tubulin loci (*TUBB*1 and *TUBB*2) was performed according to Braglia *et al.* (2021a).

Table 1. List of analysed accessions

Clone ID	Taxon	Country	State/city/region	Collection site	Donor	Year	Morphology	cp markers	AFLP	qPCR	RGS
6861	*L.* × *mediterranea*	Italy	Tuscany	Massaciuccoli Lake	WL	1954	X	X	X	X	X
7320	*L.* × *mediterranea*	Egypt	Cairo	Garden Dokki	WL	1970	X	X	X	X	X
7641	*L.* × *mediterranea*	Israel	Haifa	Hadera, Kirket Batih	WL	1972	X	X	X	X	X
9562	*L.* × *mediterranea*	Italy	Umbria, Perugia	Trasimeno Lake, Passignano	KJA	2011	X	X	X	X	X
9248	*L.* × *mediterranea*	Italy	Trentino	Trento, Loc. Alvi	WL	1999	X	X	X	X	X
9425a	*L.* × *mediterranea*	Germany	Hamburg	near Elbe	WL	2006	X	X	X	X	X
LM0027	*L.* × *mediterranea*	Italy	Campania, Neaples	Botanical Garden	CF	nd	X	X	X	X	X
9598	*L. gibba*	Italy	Sicily	nd	WL	2011	X		X	X	
7742a	*L. gibba*	Italy	Sicily, Catania	Botanical Garden	KJA	1973	X		X	X	
0190	*L. gibba*	USA	North Carolina	nd	WL	2021			X		
8124	*L. gibba*	USA	Arizona	Pima Co., Arivaca	KJA	1973			X		
7705	*L. gibba*	India	Gujarat	nd	WL	1972			X		
7796	*L. gibba*	Italy	Sicily	Catania province	KJA	nd			X		X
7922	*L. gibba*	Argentina	Buenos Aires	nd	WL	1973			X		X
9482	*L. minor*	Italy	Apulia, Bari	nd	WL	2006	X		X	X	
5500	*L. minor*	Ireland	County Cork, Blarney	5 miles East of Blarney	KJA	nd	X		X	X	X
9424	*L. minor*	Germany	Lower Saxony	Niedersachsen	WL	2006			X		
7194	*L. minor*	Uganda	Masaka	nd	KJA	1968			X		X
7753	*L. minor*	Ethiopia	Hara, Semien, Djinbar-Wans	nd	KJA	1973			X		X
8292	*L. minor*	Iran	Mazandaran, Ramsar	Ghassem Abbath	KJA	1974			X		
9495	*L. minor*	Norway	Stavanger	nd	KJA	2009			X		X

WL, Walter Lammler; KJA, Klaus J. Appenroth; CF, Cinzia Forni.

DNA barcoding analysis

The *atp*F–*atp*H and *psb*K–*psb*I plastid intergenic spacers were investigated as DNA barcoding regions by PCR amplification followed by Sanger sequencing as reported in Braglia *et al.* (2021b). Species identity was inferred from BLAST analysis against the corresponding sequences of *L. minor* (5500) and *L. gibba* (7742a) reference clones. For identification of single nucleotide polymorphisms (SNPs), sequences were aligned using the Vector NTI alignment tool, AlignX.

AFLP and data analysis

To investigate hybrid genome diversity and their relationship with parental genomes, a widely employed and cheap marker was chosen, followed by population structure analysis. The AFLP analysis was performed on all 21 duckweed clones listed in Table 1 and referring to three plant groups: *L. gibba*, *L. minor*, and putative hybrids *L. ×mediterranea*. A 50 ng aliquot of genomic DNA (gDNA) was analysed following the protocol of Vos *et al.* (1995) with modifications as described in Braglia *et al.* (2021b) considering a double DNA digestion (*Eco*RI and *Mse*I) and performing pre-selective and selective PCR amplification steps using the primers listed in Supplementary Table S1. The CE loading mixture and running protocol were prepared and adopted according to Braglia *et al.* (2023). The AFLP pherogram elaboration and processing was performed by Gene Mapper Software v. 5.0 (Thermo Fisher Scientific, Germany), allowing amplicon sizing and allele detection. For scoring all the nine primer combinations, the peak detection threshold of relative fluorescence units (RFUs) was fixed above 250 and a size range was considered between 70 bp and 450 bp. The peak size (base pairs) and height (RFUs) of each electropherogram were collected through a Microsoft Office Excel file and all the AFLP profiles were aligned according to the peak size. A binary matrix was then generated for each primer combination by scoring for presence/absence of homologous bands (0/1 respectively). FAMD (Fingerprint Analysis with Missing Data) program, v.1.31 (Schlüter and Harris, 2006) was used to estimate genetic parameters: percentage of polymorphic markers, number of fixed markers, number of species-specific alleles found in each group, within-groups mean gene diversity (HS), and between-groups gene diversity (G_{ST}; Nei, 1973). Pearson's correlation was calculated by Past 4 software v. 4.13 for Windows (Hammer *et al.*, 2001) in order to estimate the linear association between the analysed clones. A principal component analysis (PCA) was also performed using the same software. A neighbour-net diagram was constructed using SplitsTree v. 4.19.0 (Huson and Bryant, 2006) applying the Nei–Li coefficients (Nei and Li, 1979). Two thousand replicates were considered when performing the bootstrap analysis. The presence/absence matrix was also analysed by a more general Bayesian clustering approach using Structure v. 2.3.4 (Pritchard *et al.*, 2000) and a more specific approach for hybrid detection using NewHybrids v. 1.1 (Anderson and Thompson, 2002). As a first step, the initial matrix, which consisted of 1671 loci, was reduced to 694 loci by applying a minimum allele frequency of 25%, since the high proportion of loci with a low allele frequency hampered the Structure analysis from converging. The final dataset was run as diploid data with recessive alleles for the number of K clusters ranging from 1 to 5, with 50 000 burn-in steps and 50 000 additional steps. In total, 10 repetitions for each K were run. The results from Structure were analysed by the Delta K method (Evanno *et al.*, 2005) as implemented in StructureHarvester (Earl and von Holdt, 2012). Clumpp v1.1.2 (Jakobsson and Rosenberg, 2007) was used to average the 10 repetitions for each K for visualization. For the NewHybrids analysis, five datasets were created, each with 200 randomly selected loci from the Structure dataset, as NewHybrids only runs stably for a limited number of loci. After a burn-in of 10 000 steps, an additional 20 000 steps were collected. Finally, the results of the five runs were averaged.

Homoeologue-specific quantitative PCR

The following procedure was adopted in order to establish the subgenome composition of hybrids without the need for time-consuming karyotype analysis. It is an adaptation of the technique described as double-mismatch allele-specific (DMAS) qPCR for SNP genotyping (Lefever *et al.*, 2019). Instead of discriminating homo/heterozygous loci differing by one SNP, the technique is here applied to assign triploid hybrid clones to any of the two possible subgenome compositions, either two chromosome sets from *L. gibba* and one from *L. minor* (GGM) or vice versa (MMG). The assay, selectively targeting a short fragment of the *TUBB2* locus in either the *L. gibba* or *L. minor* genome, includes two slightly different primer pairs, one for each species-specific target, with similar annealing temperatures (60 °C). Primer sequences are reported in Supplementary Table S1. In the genome of hybrids, the two primer pairs are therefore homoeologue specific. The quantitative determination of each target subgenome by the specific primer pairs is proportional to the quantification cycle (Cq), the PCR cycle at which the fluorescent signal can be detected over a threshold. Amplification of the non-target homoeologue subgenome may occur only at very high Cq. The principle is that, in parallel PCR amplifications, absolute ΔCq between the two primer pairs (Cq_{minor}–Cq_{gibba}) is maximal for both target species *L. minor* and *L. gibba*, homozygous at this locus, and close to zero for homoploid hybrids, where both subgenomes are equally present, behaving as heterozygous. Intermediate subgenome compositions in triploid hybrids should produce higher or lower $\Delta Cq_{(minor-gibba)}$ values with respect to the diploid hybrids, respectively, depending on the prevalent subgenome.

PCR amplification was performed in a CFX-connect qPCR system (BIORAD) with hard-shell 96-well plates (BIORAD). Each reaction was carried out with 4 µl of master mix (Titan HotTaq EvaGreen, BIOATLAS), 0.5 µl of each primer (from a 100 µM stock), and 3 µl of DNA (2 mg ml^{-1}), in a final volume of 50 µl. The two-step amplification profile used was the following: initial denaturation, 15 min at 95 °C, followed by 39 cycles of 15 s at 95 °C/60 s at 60°C, and final denaturation by a 0.5 °C step increase up to 95 °C for melting curve analysis. Primers are listed in Supplementary Table S1.

The threshold for Cq determination was set by the regression method. Primer specificity and amplification efficiency were first tested on serial dilutions (2, 0.2, and 0.02 mg ml^{-1}) of gDNA purified from each parental species, *L. gibba* (clones 7742a and 9598) and *L. minor* (clones 5500 and 9482), accurately quantified fluorometrically in duplicate, diluted to 2 mg ml^{-1}, and measured again. Artificial hybrid genomes were then obtained by independently mixing gDNA from *L. minor* 5500 (M) with *L. gibba* (G) 7742a and *L. minor* 9482 (M) with *L. gibba* (G) 9598. Equimolar (1:1) DNA ratios (MG mix 1-2) mimicked homoploid hybrid genomes, while two unbalanced mixtures in 1:2 molar ratios (GGM mix 1-2 and MMG mix 1-2) simulated triploid hybrid genomes. The method was first validated by parallel PCR amplifications with the two primer pairs on the six artificial hybrid genomes. For statistical significance, ΔCq values of each group (MG, MMG, and GGM) were averaged and analysed by one-tailed ANOVA. The DNA of the two target species and the seven hybrid clones was then tested in triplicate in at least two independent experiments, by the same parallel PCR amplification. For each sample, ΔCq values of all nine replicates, excluding outliers (±2Cq from the mean), were mediated and plotted. The difference of the Cq means between triploid and diploid *Lemna* clones was tested by Student's *t*-test and ANOVA.

Morphological analyses

Morphological analyses were carried out on fronds of each of the seven putative hybrid *Lemna* clones assigned to the hybrid taxon *L. ×mediterranea*, that were grown for 3 weeks, in 600 ml glass beakers filled with mineral water of known composition (Supplementary Table S2), close to that found in many natural water bodies. Plants were grown in the laboratory, under the same temperature and light conditions, although not strictly controlled. For comparison, two diploid clones of each parental species *L. gibba* (clones 7742a and 9598) and *L. minor* (clones 5500 and 9482), of European origin, were similarly grown and analysed. The entire

set of beakers was placed near the window to be exposed directly to natural light respecting the summer seasonal photoperiod.

To morphologically describe the putative hybrid clones, 10 specimens of each clone were randomly collected in parallel with those of the parental species, for a total of 110 fronds. Each of these specimens was observed and photographed in dorsal, ventral, and lateral position under a stereomicroscope (Olympus SZX2-ILLT) equipped with an Olympus OM-D EM-5 camera. Morphological traits of each specimen were analysed and measured using the image-processing program ImageJ software v. 1.53t (Schneider *et al.*, 2012). The analysed traits were selected after consulting reference literature related to *Lemna* species (e.g. Landolt, 1980, 1986; Ceschin *et al.*, 2016; Bog *et al.*, 2020b). They included both quantitative and qualitative morphological characters, as listed in Supplementary Table S3 (Fig. 1). If the specimen consisted of contiguous fronds (colony), the traits were analysed only on the mother frond; it was complete with root, and was the largest and placed above all the other fronds.

Scanning electron microscopy observations

To better observe other potential morphological traits useful to further differentiate the *Lemna* groups, some fronds were observed by SEM. Specifically, from each of the four *Lemna* groups identified by genome size measurement, 10 specimens were randomly taken and fixed overnight at 4 °C in a mixture of 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer. The next day, specimens were thoroughly washed in the same buffer and post-fixed in 1% buffered osmium tetroxide for 90 min at 4 °C. After thorough washing, first in 0.1 M cacodylate buffer and then in double-distilled water, the specimens were dehydrated through a graded ethanol series (15, 30, 50, 75, 85, 95, and 100%) and dried in a critical point dryer (CPD 030 unit, BalTec, Balzers, Liechtenstein). Specimens were mounted on aluminium stubs using double-sided carbon discs, and gold sputtered using a K550 sputter coater (Emithech, Kent, UK). The specimens were then observed and microphotographed by a scanning electron microscope (Gemini 300, Carl Zeiss AG, Jena, Germany).

Analysis of plant growth and biochemical parameters

Plant growth and biochemical analyses were performed on cultures grown under controlled and axenic conditions in 150 × 75 mm (d×h) Petri dishes (Corning Inc., Corning, NY, USA) that contained 150 ml of freshly prepared, liquid SH medium (pH adjusted to 5.5) and 0.5% sucrose. Plants were cultivated at a 16 h day photoperiod under 100 µmol photons m^{-2} s^{-1} at 25 ± 2 °C. Experimental cultures were started by inoculating 30 colonies with 2–3 fronds each. Growth measurements and biochemical analyses were carried out after 7 d. All cultures were set up in quintuplets.

Frond vascular organization

To determine frond vein numbers, 10 duckweed colonies, with two/three fronds each, were washed with deionized water and cleared with 70% ethanol for 3 weeks prior to observations using a Nikon stereomicroscope (Nikon SMZ1000) equipped with a Nikon digital camera (DS-5M). Duckweed colonies were observed under bright and dark field conditions at ×20 and ×10 magnification.

Stomatal traits

To characterize stomatal traits, for each *Lemna* clone, three colonies with two or three fronds were washed in demineralized water and immersed in 70% ethanol solution for 3 weeks to remove any pigmentation. Stomata features were examined and photographed using a Nikon microscope (ECLIPSE 80i) equipped with a digital camera (Nikon DS-5M; Nikon Instruments Inc.). Stomatal density and stomata size were determined by analysing images of four different microscopic fields (0.95 μm^2) for each mother frond of three colonies/clone at a magnification of ×20. Fields were selected in the regions located between the main vein and the closest secondary vein (two sectors to each side of the main vein) (Supplementary Fig. S1). Stomatal density was expressed as stomata number/area of one microscopic field (area). The number and the size of stomata were measured using the LeafNet software (Li *et al.*, 2022). Parameters were adjusted by setting 'Stained Denoiser' for the Image denoizer function and 'StomaNet Universal' for the Stoma detector function. Stoma minimum size was set to 300 μm^2.

Analyses of growth parameters

For fresh and dry weight measurements, all plants from each tested clone were sieved out of the medium, dry blotted, and either weighed immediately (FW) or dehydrated at 60 °C for 72 h and then weighed (DW).

The mean single frond FW of each clone was estimated by measuring the total biomass of each experimental culture and dividing by the corresponding total number of fronds (including daughter fronds when still attached to the mother) previously counted using the ImageJ image processing program (Schneider *et al.*, 2012).

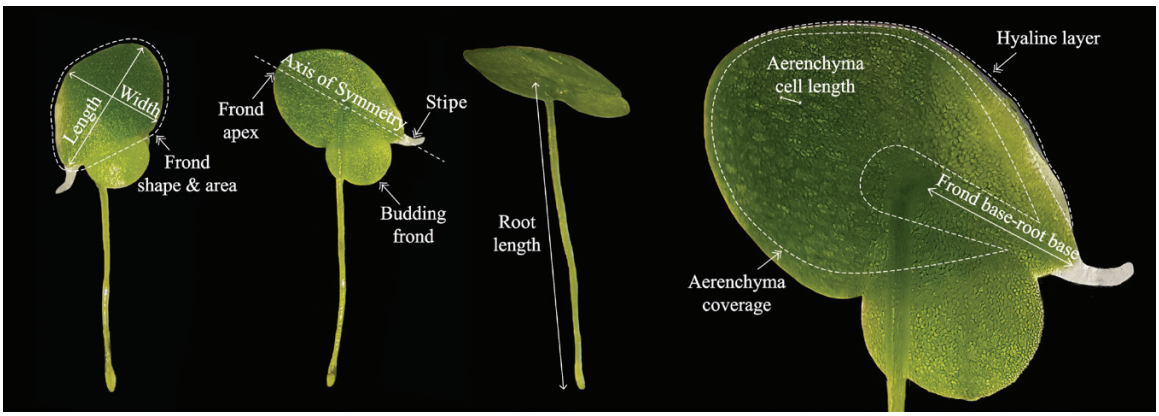

Fig. 1. Illustration of the morphological traits analysed.

Relative growth rate

The relative growth rate (RGR) of each *Lemna* clone was measured after 7 d and calculated using the following formula: $RGR=\ln(DW_f-DW_i)/T_f-T_i)$, where: DW_f=final DW (g), DW_i=initial DW (g), T_f=total incubation period (d), and T_i=initial time (d). The results were expressed as g g^{-1} d^{-1}.

Determination of chlorophyll and carotenoid contents

Fresh fronds (0.1 g) were ground into powder with liquid nitrogen, and then homogenized with 80% (w/v) cold acetone, and centrifuged at 5000 *g* for 10 min. The absorbance of the supernatant was measured at 663, 646, and 470 nm. Chl *a*, Chl *b*, and carotenoid contents were determined using the equations described in Lichtenthaler (1987). The results were expressed in milligrams of chlorophyll or carotenoids per gram of plant tissue FW (mg g^{-1} FW).

Measurement of protein content

Lemna fronds (0.1 g FW) were ground in liquid nitrogen with a mortar and pestle. The proteins were then extracted at 4 °C with a cold 0.5 M potassium phosphate (pH 7.0) buffer containing 0.1% ascorbic acid, 0.1% Triton X-100, 1 mM EDTA, and 7.5% polyvinylpyrrolidone. The homogenate was centrifuged at 4 °C for 20 min at 12 000 *g*. The total soluble proteins were quantified according to Bradford (1976) using BSA as standard. The results were expressed in milligrams of proteins per gram of plant tissue FW (mg g^{-1} FW).

Statistical analyses (for morphological data)

All statistical analyses were performed using R software, version 4.2.1. (R Core Team, 2022). All selected morphological traits and datasets comprising growth analysis and biochemical parameters were analysed using PCA, and biplots were made considering PC1 and PC2 using either ggfortify or the factoextra packages of the R software (Tang *et al.*, 2016; Kassambara and Mundt, 2020). For plant growth and biochemical data analyses, the cos2 values were considered. A high cos2 value indicates that a higher impact of the Wtraits was compared between clones using ANOVA. Where assumptions of normality and homoscedasticity were not met, a non-parametric test was conducted (Kruskal–Wallis). Qualitative traits were analysed by calculating contingency tables and performing Pearson's χ^2 tests. Boxplots and mosaicplots were made using ggplot2 package v. 3.4.2 (Kassambara, 2023). Specifically, for multivariate analyses 'ade4' and 'vegan' R packages were used (Dray and Dufour, 2007; Oksanen *et al.*, 2020) and the significance level was set to $P<0.05$. The post-hoc Tukey's honest significant difference test (Tukey HSD) was run to adjust *P*-values for multiple comparisons to determine which samples have significantly different means in paired sample comparison.

Results

Molecular characterization of the additional, putative hybrid, Lemna *clone LM0027*

The hybrid origin of six of the seven clones analysed in this study from an interspecific cross between *L. minor* and *L. gibba* was previously suggested, relying on TBP profiling and plastid marker sequences. Their amplicon profiles for both introns were in fact additive with respect to the parental species, while no contribution from any other *Lemna* species was evident (Braglia *et al.*, 2021b). The six specimens were all identified as *L. gibba* by their collector E. Landolt, by morphological analysis. An additional clone included in this study, LM0027, classified as *L. minor* by its collector (C. Forni), was then analysed by the same markers. However, the same TBP pattern as that observed for the other six hybrid clones, which merges profiles of the two putative parents *L. minor* and *L. gibba*, was observed for LM0027. Every putative hybrid clone is then heterozygous at all six β-tubulin loci (Braglia *et al.*, 2021b). LM0027 groups together with the other six putative hybrid clones by cluster analysis of TBP markers, well separated from the clusters of each parental species (seven clones each, from different geographic areas were chosen as representative of the intraspecific genetic diversity, Supplementary Fig. S2). Sequences of both intronic regions of the β-tubulin locus *TUBB*1 (Supplementary Fig. S3), amplified by specific primers, confirmed also for clone LM0027 the identity of each homoeologous allele with the corresponding parental species, upon BLAST DNA analysis against the genome sequence of *L. gibba* 7742a and *L. minor* 9252, respectively (www.lemna.org/blast; accessed on 27 April 2023). BLAST DNA analysis of the nucleotide sequences obtained for the two plastid markers *psb*K–*psb*I (512 bp) and *atp*F–*atp*H (529 bp) (Supplementary Fig. 1) permitted the establishment of the parentage of the newly investigated clone LM0027, which turned out to have plastid marker sequences matching those of *L. minor*, and are almost identical to the four previously analysed hybrid clones 7641, 6861, 9562, and 7320 (one SNP), thus having *L. minor* as the maternal parent. For the two remaining clones, 9248 and 9245a, their origin from the reciprocal cross was previously assumed from their plastid marker identity to *L. gibba* sequences (Braglia *et al.*, 2021b).

Genome size estimation and subgenome composition of hybrid clones

Plant interspecific hybrids are in most cases polyploid but can be also diploid (homoploid) when the two different subgenomes are shared within the same nucleus without an increase in chromosome number (Abbott *et al.*, 2010). The relative genome size (RGS) of each *L. ×mediterranea* clone was then assessed by flow cytometry in comparison with that of the parental species and used as a proxy of ploidy (Table 2; raw data for flow cytometry are reported in Supplementary Table S4). The five clones with *L. minor* as the maternal parent showed an average RGS of 0.54, exactly intermediate between the values of the two diploid parental species (0.46 *L. minor*, 0.64 *L. gibba*), perfectly fitting what was expected for a homoploid hybrid. Conversely, the RGS of the two clones having *L. gibba* as the maternal parent, 0.84, was ~1.5× larger, suggesting a triploid state. This led us to conclude that the analysed clones belong to two different cytotypes, most probably a homoploid and a triploid, respectively. Both kinds of hybrids, although rarer than tetraploids or hexaploids, may occur in plants and are generally considered as bridges toward higher ploidy levels, eventually

Table 2. Genetic structure of seven *L.* ×*mediterranea* clones (hybrids) and parental species

ID	Taxon by TBP	Plastid donor	RGS	Ploidy	Subgenome composition
7796	*L. gibba*	*L. gibba*	0.65	2*n*	GG
7922	*L. gibba*	*L. gibba*	0.62	2*n*	GG
5500	*L. minor*	*L. minor*	0.46	2*n*	MM
7194	*L. minor*	*L. minor*	0.45	2*n*	MM
9495	*L. minor*	*L. minor*	0.46	2*n*	MM
7753	*L. minor*	*L. minor*	0.46	2*n*	MM
6861	*L.* ×*mediterranea*	*L. minor*	0.54	2*n*[a]	MG
9562	*L.* ×*mediterranea*	*L. minor*	0.54	2*n*[a]	MG
LM0027	*L.* ×*mediterranea*	*L. minor*	0.54	2*n*[a]	MG
7320	*L.* ×*mediterranea*	*L. minor*	0.54	2*n*[a]	MG
7641	*L.* ×*mediterranea*	*L. minor*	0.54	2*n*[a]	MG
9248	*L.* ×*mediterranea*	*L. gibba*	0.84	3*n*[a]	GGM
9425a	*L.* ×*mediterranea*	*L. gibba*	0.84	3*n*[a]	GGM

[a] Deduced from genome size; G and M refer to *L. gibba* and *L. minor* subgenomes, respectively; RGS, relative genome size.

leading to hybrid speciation (Bretagnolle and Thompson, 1995; Tayale and Parisod, 2013; Mason and Pires, 2015).

Triploid hybrids may have two different subgenome compositions—MMG or GGM—depending on the donor of the diploid gametes. Further analysis was then conducted in order to determine the subgenome composition of each hybrid clone, by a modification of the DMAS qPCR technique (Lefever *et al.*, 2019). Genomic DNA of two clones for each parental species, the seven hybrid clones, and six artificial hybrid genomes (MG1 and 2, GGM1 and 2, and MMG1 and 2) obtained by mixing gDNA of *L. gibba* and *L. minor* in different proportions was amplified in parallel with two homeologue-specific primer pairs and the $\Delta Cq_{(minor-gibba)}$ values were recorded. The specificity of the two primer pairs is shown in Fig. 2A where representative amplification plots are shown. Mean $\Delta Cq_{(minor-gibba)}$ values for *L. gibba* 7742a and *L. minor* 5500 were equal to 10.84 and −12.83, respectively (mean of triplicate technical repetitions, three independent experiments). Small but significant differences in ΔCq values were observed between artificial hybrid genomes made up of three different gDNA ratios, for test validation. As expected, DNA mix MG1 and MG2, with a 1:1 composition of *L. minor* and *L. gibba* DNA, showed very low ΔCq values (mean= −0.47 ± 0.05). Both unbalanced 1:2 DNA mixtures, GGM and MMG, gave mean ΔCq values significantly ($P<0.01$ by ANOVA calculation and Tukey HSD) higher (0.42 ± 0.04) or lower (−1.25 ± 0.11) than MG, respectively, with a ΔΔCq between the triploid-like DNA mix and the diploid-like mix of +0.89 and −0.78, respectively, a difference sufficient to discriminate between the two genotypes. Mean ΔCq values obtained for each natural hybrid DNA were then plotted (Fig. 2B). Mean ΔCq values obtained for the triploid group (0.75 ± 0.21 and 0.52 ± 0.32) were significantly higher (ANOVA, $P<0.05$) than the mean ΔCq of the diploid group, close to 0 (−0.080 ± 0.14), giving ΔΔCq values of 0.83 and 0.59, respectively, which indicates that triploids have a GGM genotype. This conclusion is also supported by the observation that the measured RGS for these two triploid clones, 0.84 (Table 2), is closer to the theoretical genome size calculation for GGM hybrids (0.86) than for MMG (0.77), based on RGS of the parental species. The small discordance between ΔCq values of hybrid clones and the corresponding artificial genome mixtures is likely to be due to inaccuracy of quantification of the DNA preparation used to make admixtures.

Genome diversity by AFLP analysis

AFLP analysis of seven clones for each group (parents and putative hybrids) already analysed by TBP provided confirmation of hybridization at the whole-genome scale. In this regard, the AFLP analysis yielded 1671 markers, 98% of which were polymorphic considering 21 duckweed clones. The number of polymorphic markers within the groups of clones of *L. minor* and *L. gibba* was 896 (54%) and 856 (51%), respectively, significantly higher than those estimated within the third group of clones (*L.* ×*mediterranea*) that revealed only 21% polymorphism. Accordingly, the lowest number of species-specific alleles, 37, was found in this latter group, compared with 456 and 354 species-specific alleles detected for *L. minor* and *L. gibba* groups, respectively, reflecting the conspicuous number of loci shared between the putative hybrid group and both parents. In addition, mean genetic diversity estimated within taxa (HS) was 0.1059 in *L. gibba*, 0.0750 in *L. minor*, and 0.0221 in *L.* ×*mediterranea*. Conversely, the between-population gene diversity (G_{ST}, Nei, 1973) value was significantly higher ($P<0.05$) when comparing each of the *L. minor* and *L. gibba* groups (0.2638), than when comparing *L.* ×*mediterranea* with either of the two parents (0.1224 and 0.1160 with *L. minor* and *L. gibba*, respectively). In this context, the diagram of the Pearson's linear correlation (Fig. 3) estimated among all analysed clones returned the highest significantly ($P<0.05$) recorded values among the accessions of *L.* ×*mediterranea*, forming a group of clones

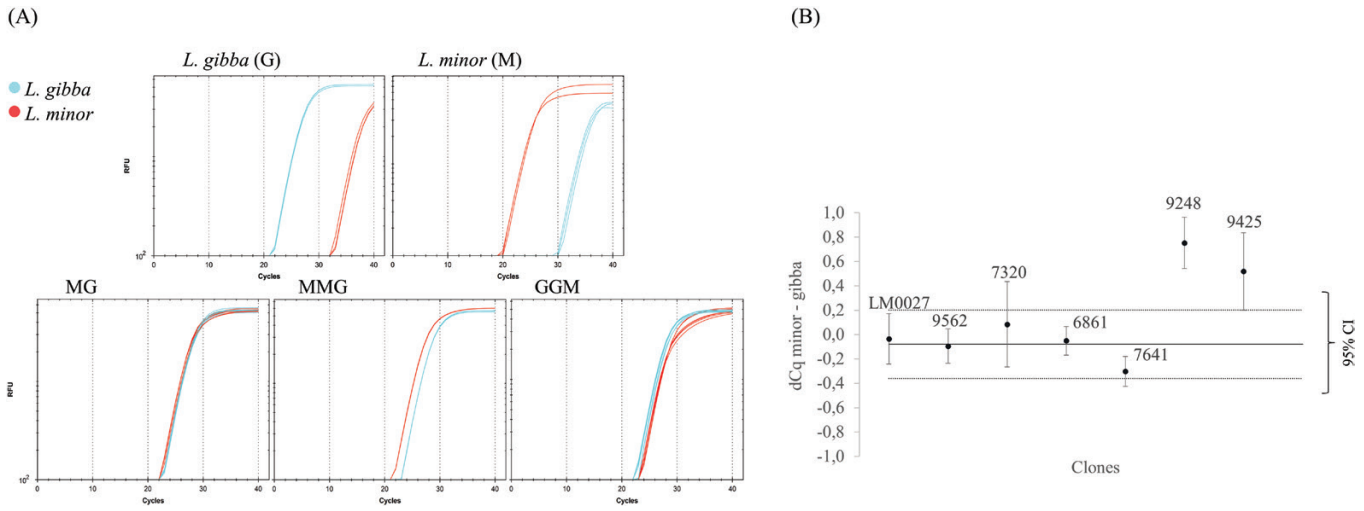

Fig. 2. Homeologue-specific qPCR. (A) Representative PCR amplification plots of the parental species' DNAs and their mixtures in different proportions (upper panel). Colours indicate the specific target of the primer pair used. (B) Scatter plot of the Cq differences between the perfect match and mismatch reactions for each *L. ×mediterranea* clone (*n*=3). Horizontal lines indicate the mean value of the five diploid samples and the 95% confidence interval (±2SD).

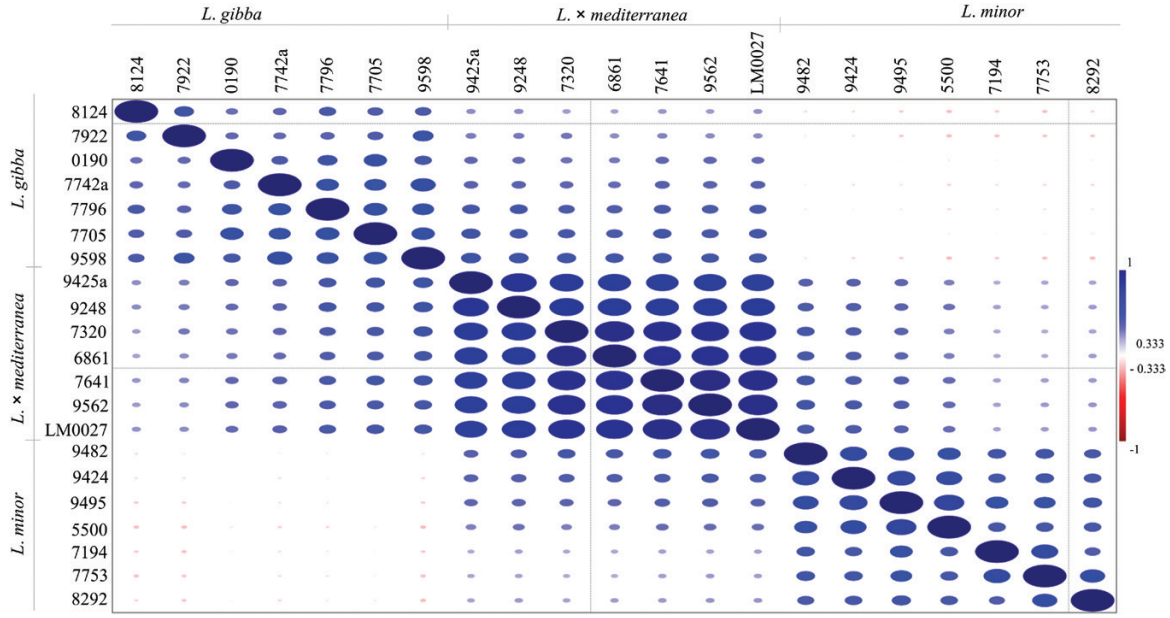

Fig. 3. Pairwise Pearson's correlation matrix, comparing parental and putative hybrid genotypes. Clone numbers refer to those reported in Table 1.

strongly related to each other, while the lowest correlation was assessed among *L. gibba* clones.

The total variance accounted for by each component of the PCA (PC1 37% and PC2 21%) in Supplementary Fig. S4 grouped the analysed clones into three distinct, non-overlapping, and well-defined clusters, further highlighting that a representative estimate of the total genetic variability (52%) can be attributed to variability detected among groups.

Neighbour-net analysis (Fig. 4) also supported the existence of differentiated groups of individuals. Despite the evident reticulation, three diverging groups were formed by a strongly supported split (bootstrap values: 87, 90, and 100%): two of these correspond to the *L. minor* and *L. gibba* groups of clones, considered as the parental species involved in the cross, whereas a third group, located between the other two, represents an isolated entity formed by the seven clones of *L. ×mediterranea* considered as the derived hybrid. A substantial amount of reticulation occurred in particular within parental groups, reflecting the geographic partition (America, Europe, India, and Africa) (Table 1) characterizing the selected clones considered

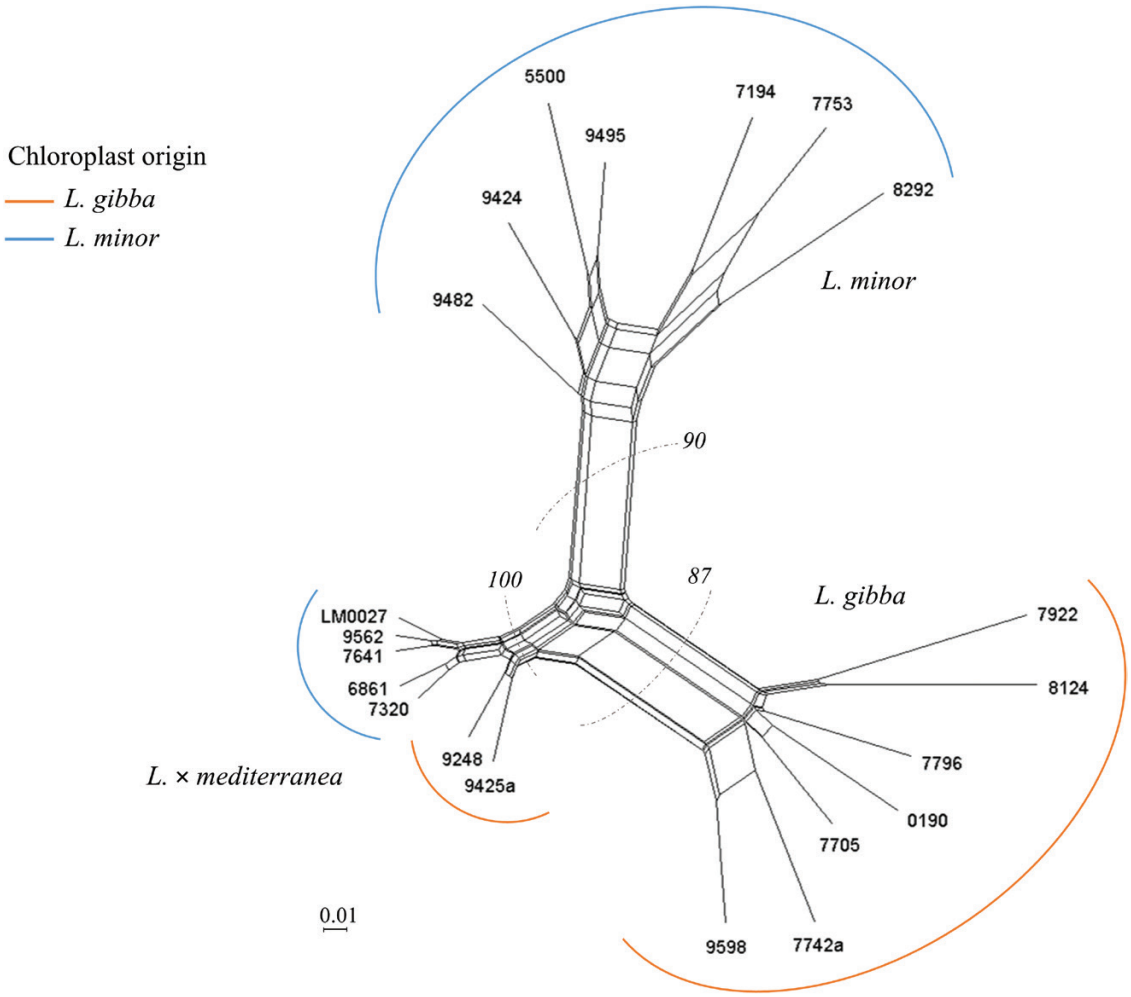

Fig. 4. Phylogenetic network (NeighborNet) constructed on the AFLP patterns. Bootstrap values are given for the main clusters. Grouping by colours is made according to the maternal parentage determined by plastid markers.

as representative of the two species. Moreover, within the *L.* ×*mediterranea* group, two sub-branches were observed, in accordance with the already documented different chloroplast origin (coloured lines in Fig. 4) and subgenome composition (GGM, triploid) of two of the seven hybrid clones (9248 and 9425a).

The Structure analysis with the reduced dataset (694 loci) strengthens the origin of the putative hybrid individuals. According to the delta K method, the highest probability of dividing the dataset into two clusters correlates with the two parent species. The putative hybrid individuals show an ~50% affiliation for each of the two clusters of the parent species (Fig. 5) and they would even be assigned to their own cluster if three clusters were assumed. Strikingly, the two individuals with the GGM genome composition show a further deviation from the hybrid cluster when assuming four clusters.

These results are further supported by the analysis with NewHybrids (Supplementary Table S5). All putative hybrid individuals were categorized as F_1 hybrid crosses between *L. gibba* and *L. minor*. There was no assignment to the F_2 or either backcross category.

Morphological diversity

To compare morphological diversity between *L.* ×*mediterranea* and the parental species, two diploid clones of Mediterranean origin, more closely related to the hybrids, were chosen as representative of each parental species *L. gibba* (GG) and *L. minor* (MM). Morphological analysis of 10 fronds of each clone of the two parents, as well as the two hybrid cytotypes, showed that the two hybrid classes, triploid (GGM) and homoploid (MG), are distinct not only genetically but also morphologically, despite large trait overlaps with one another and with parental species (Fig. 6A).

Most of the morphological traits considered are useful in differentiating between the four *Lemna* groups (Supplementary Table S6). Such differences are more marked between the two parental species than between them and the two hybrid

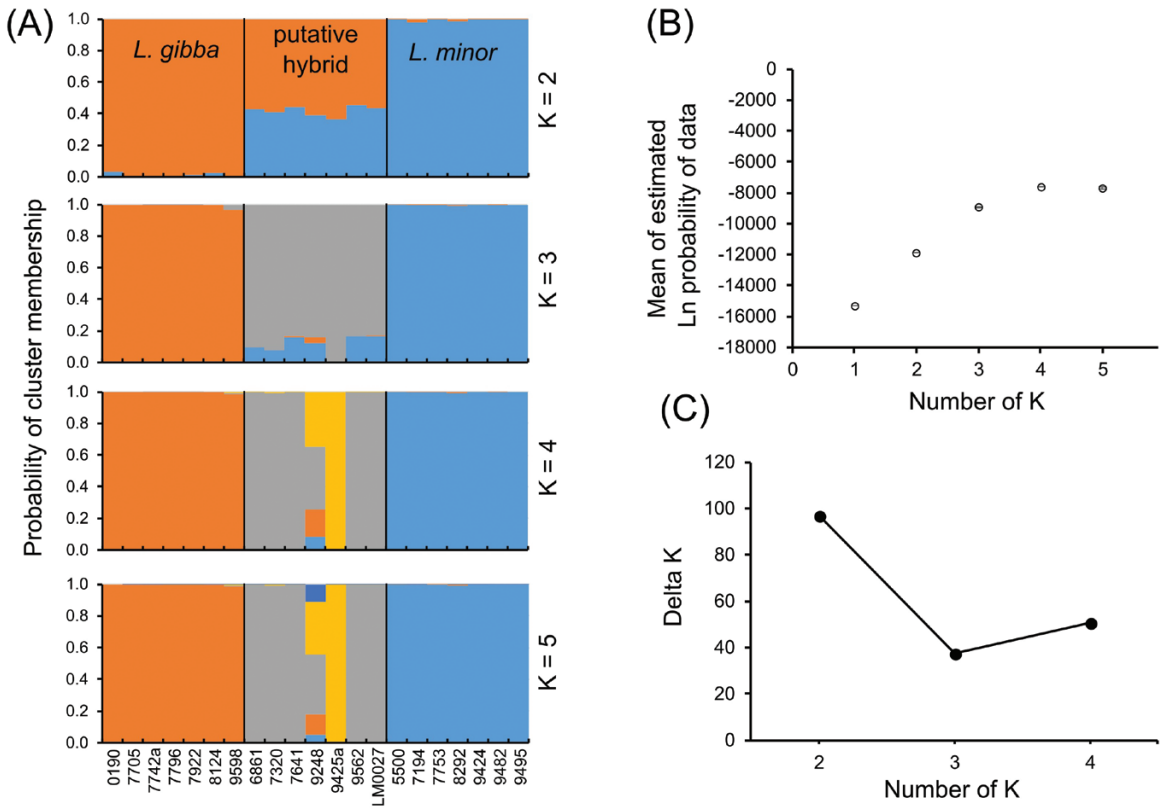

Fig. 5. Results of the Structure analysis based on the reduced AFLP dataset. (A) Cluster membership of the 21 investigated clones for the number of clusters K=2–5. (B) Mean Ln probability values and their SDs from the 10 independent Structure runs for K=1–5. (C) Results of the Delta K method, showing the highest value for K=2.

cytotypes. There are significant differences between the two hybrid cytotypes, particularly in quantitative traits (Fig. 6B), while for qualitative traits, there are several overlaps (Fig. 6C; Supplementary Table S6).

All the quantitative morphological traits considered, except aerenchymal cell length, showed significant differences between the two hybrid cytotypes (Fig. 7; Supplementary Table S7). GGM fronds differed significantly from MG in larger surface (7.90 mm^2 versus 6.53 mm^2), greater width (2.87 mm versus 2.54 mm), higher aerenchyma abundance (72.50% versus 54.60%), longer roots (5.87 mm versus 2.81 mm), and a larger number of veins (5.00 versus 3.8), on average. Conversely, GGM and MG did not show any significant difference from the maternal species *L. gibba* and *L. minor*, respectively, in relation to some quantitative parameters (frond area, frond length/width ratio, root length); in addition, GGM did not differ significantly from *L. gibba* for frond width (Fig. 7; Supplementary Table S7).

Although there were significant differences in qualitative morphological traits between the four genetically distinct groups of *Lemna* clones studied, several overlaps for these traits were found between the two hybrid cytotypes (Fig. 8). Thus, this set of traits contributes less to differentiating the two hybrid cytotypes. With specific reference to the frond shape, the pyriform shape occurred in all the groups except for *L. minor*, which had a predominantly obovate shape; a stocky rhomboid shape was absent only in *L. gibba*. A frond edge completely hyaline all round was characteristic of *L. gibba* and GGM, while it was very sporadic in *L. minor* and MG. A total absence of the hyaline edge was mainly found in both *L. minor* and MG, while it was sporadic in *L. gibba*. Elongated stipes, stolon-like appendages connecting daughter and mother fronds, occurred in all *Lemna* groups, except for *L. minor*. Only in *L. gibba* was aerenchyma dispersed throughout most of the frond area where generally it reached the edge, while in the other groups it was mostly in an upper-central position. Furthermore, only in a few individuals of the MG hybrid and *L. minor* was a centrally located aerenchyma found. The papules trait also exclusively differentiated *L. gibba* from the other *Lemna* groups since papules were always absent in *L. gibba* and generally most evident in the MG cytotype, followed by *L. minor*, and finally the GGM cytotype.

Frond vascular organization

Differences in the simplified vascular tissues were observed comparing cleared frond specimens across the 11 *Lemna* clones considered. In Fig. 9, representative stereomicroscope images of fronds show visible interior veins within the body of the thallus. In particular, in *L. minor* 5500, a central vein and two

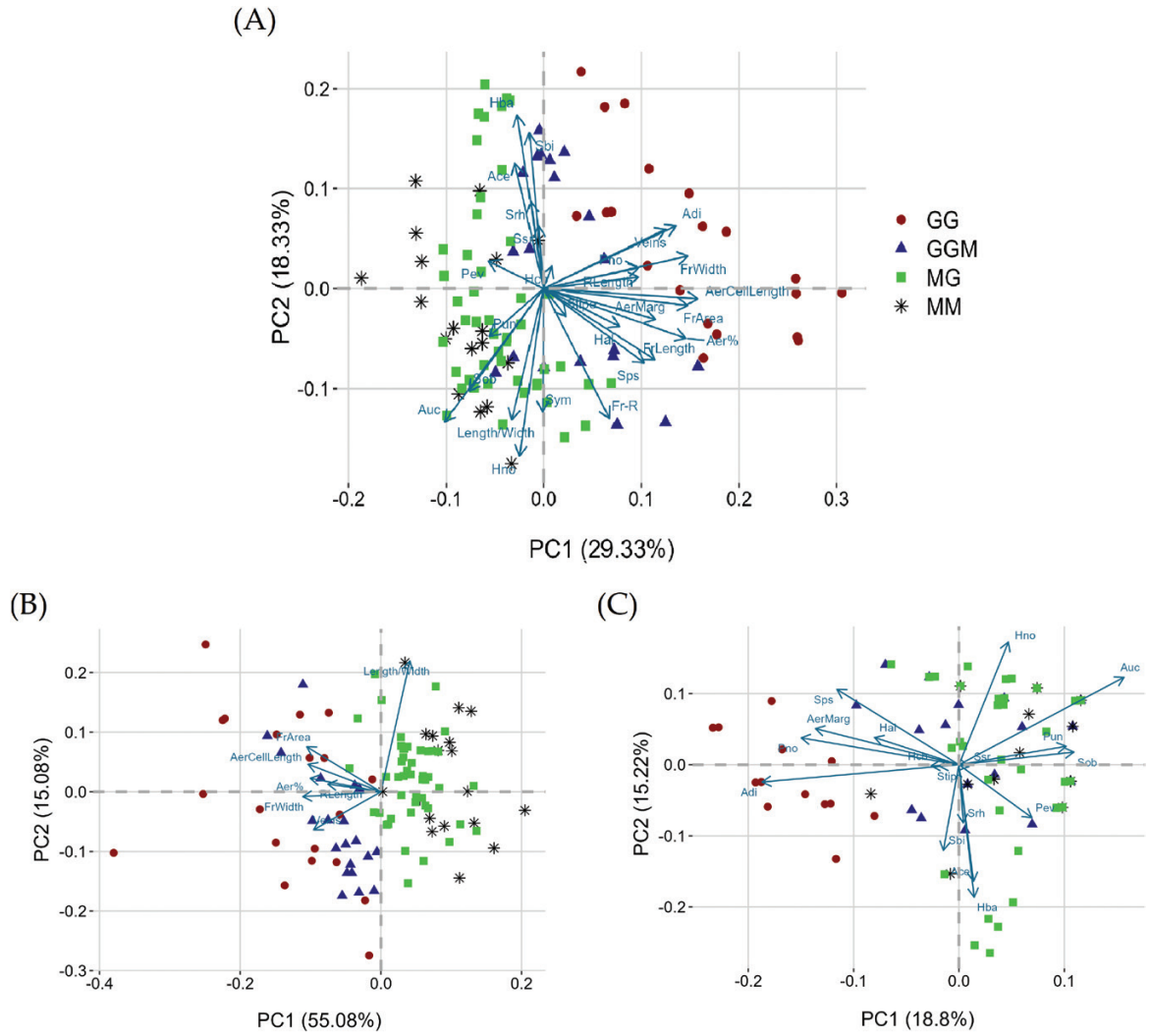

Fig. 6. Principal component analysis (PCA) of morphological traits. (A) PCA based on all considered morphological traits for the 11 investigated clones (n=10 specimens for each clone). Quantitative (B) and qualitative (C) morphological traits statistically significant from ANOVA and χ^2 tests, respectively, are shown. Acronyms for quantitative traits: FrLength, frond length; FrWidth, frond width; Length/Width, frond length/width ratio; FrArea, frond area; Veins, vein number; RLength, root length; Fr-R, distance frond base–root base; Aer%, aerenchyma percentage coverage in frond; ArCellLength, aerenchymatic cell length. For qualitative traits: Sbi, bilobate irregular frond shape; Sob, obovate shape; Sps, pear-shaped; Srh, rhomboid shape; Ssr, stocky rhomboid shape; Hal, all around hyaline frond edge; Hba, basal hyaline edge; Hcb, central–basal hyaline frond edge; Hno, no hyaline hyaline edge; Ace, central aerenchyma position; Adi, dispersed aerenchyma; Auc, upper–central aerenchyma; AerMarg, aerenchyma reaching the frond edge; Pno, absent papules; Pun, unclear papules; Pev, evident papules.

lateral veins arising from the point of root attachment were present, while in *L. gibba* 7742a five veins branched off from the node as reported in the literature (Landolt, 1986; Bog *et al.*, 2019). MG hybrid cytotypes (e.g. 7641 and LM0027) exhibited from three to four veins per frond while GGM hybrid cytotypes predominantly revealed five veins like the maternal parent species.

Stomatal traits

Stomatal density can be an indicator of the level of adaptation to environmental conditions. Stomatal size and density are dramatically impacted by growth environment factors, including light intensity, water stress, and CO_2 concentration elevation. Measurement of stomatal size and density is summarized in Fig. 10. In *L. minor*, stomatal density and size were correlated as the observed reduced stomatal density corresponded to a lower stomatal size. In particular, stomatal density and size in *L. minor* were significantly lower when compared with *L.* ×*mediterranea* MG. The highest stomatal density was observed in the diploid MG. *Lemna gibba* and the triploid *L.* ×*mediterranea* GGM did not significantly differ in stomatal density, neither did *L. minor* and MG. *Lemna minor* presented the smallest stomatal size, and GGM showed the highest. This is consistent

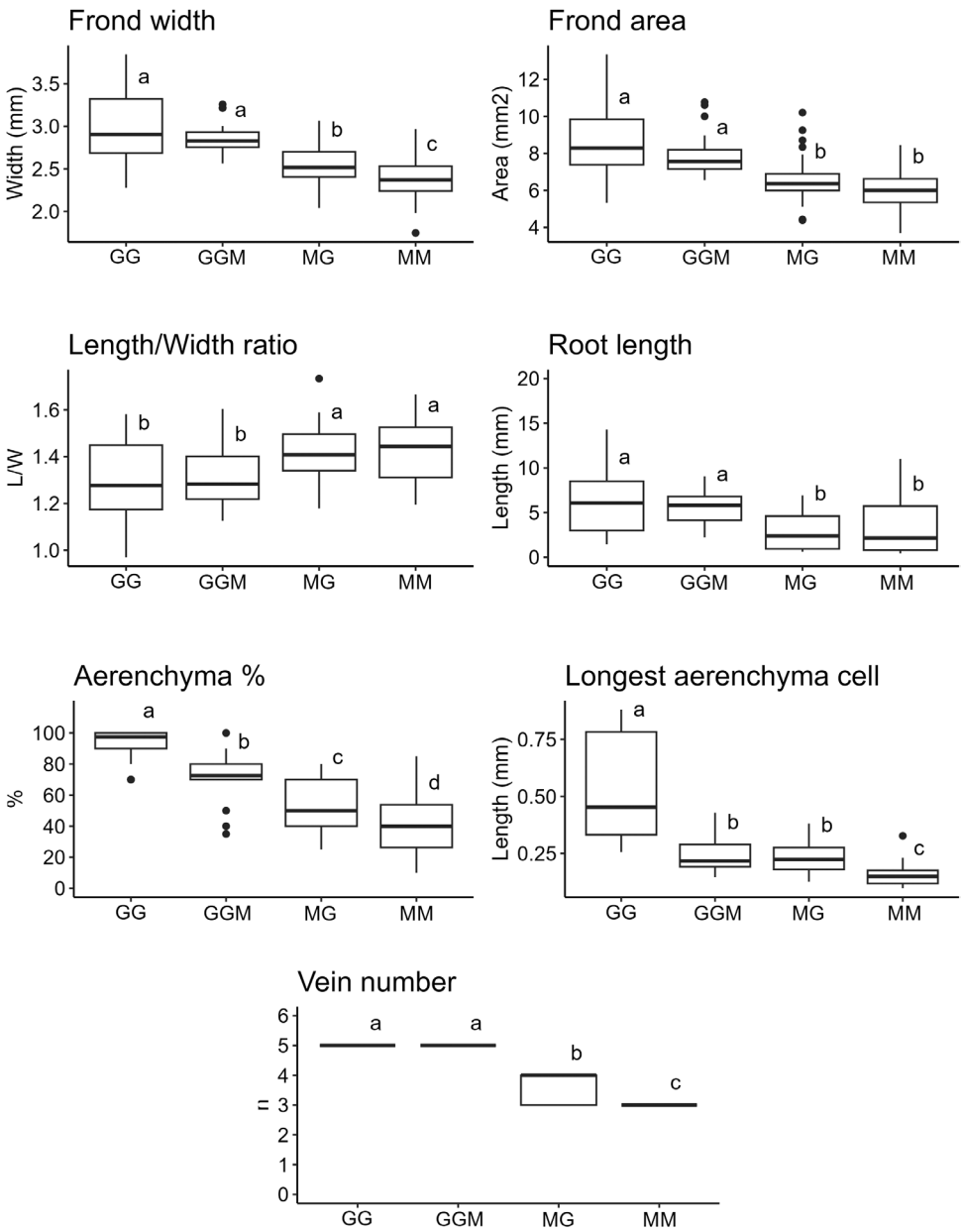

Fig. 7. Differences among the four *Lemna* groups in relation to quantitative traits found to be significant by ANOVA test (n=10). In each graph, boxplots with different letters represent significant differences at P-value ≤0.05.

with the fact that GGM clones have a higher DNA content than diploids, which usually correlates with cell size (McGoey *et al.*, 2014; Da Silva *et al.*, 2020).

Plant growth and biochemical characterization

The biochemical analysis (pigment and protein content) and plant growth parameters (RGR and frond FW) showed that the two *L. ×mediterranea* cytotypes exhibit their own independence and greater association with one of the parental species, as shown by PCA (Fig. 11). PCA performed on the dataset captured 89.7% of the cumulative variance using the parameters influencing the first two principal components. The outcomes of PCA clearly discriminated *L. minor* (MM) and homoploid hybrid clones MG from *L. gibba* (GG) and GGM triploid hybrids. The profile of *L. minor* and MG clustered in a PC1-negative direction while *L. gibba* and GGM clustered in a PC1-positive direction.

Measured parameters are summarized as boxplots in Fig. 12. No significant differences were found between *L. gibba* and GGM triploid hybrids in photosynthetic pigment content. The Chl *a* content differed significantly between *L. gibba* and GGM

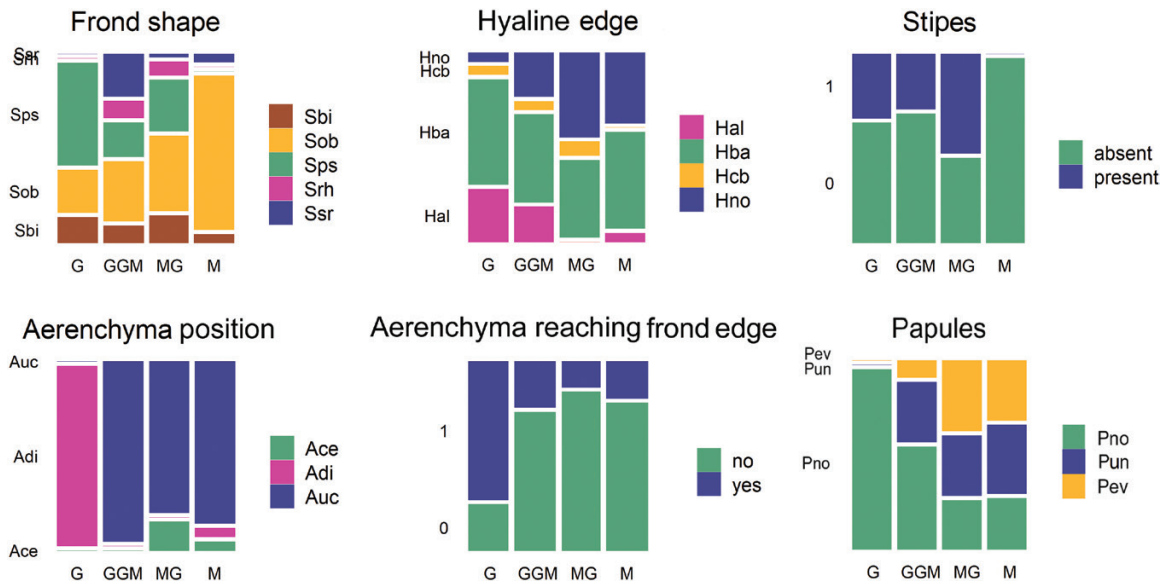

Fig. 8. Differences among the four *Lemna* groups in relation to qualitative traits found to be significant by χ^2 test (Mosaicplots) (n=10). Acronyms for frond shape (Sbi, bilobate irregular shape; Sob, obovate; Sps, pear-shaped; Srh, rhomboid; Ssr, stocky rhomboid); hyaline frond edge (Hal, all around hyaline; Hba, basal; Hcb, central–basal; Hno, no hyaline); stipes present or not (absent); aerenchyma position (Ace, central; Adi, dispersed; Auc, upper–central aerenchyma); aerenchyma reaching (yes) or not (no) the frond edge; and papules (Pno, absent; Pun, unclear; Pev, evident).

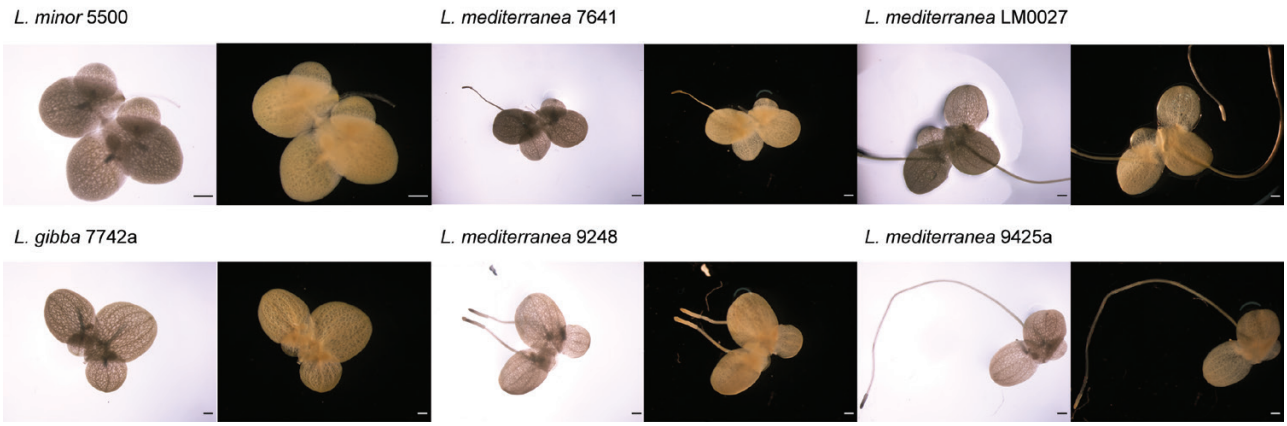

Fig. 9. Representative stereomicroscope images of cleared frond colonies for the determination of vein number per frond in the two parental species, *L. minor* 5500 and *L. gibba* 7742a, and in both *L.* ×*mediterranea* cytotypes, homoploid (MG-7461 and MG-LM0027) and triploid (GGM-9248 and GGM-9425a). Frond colonies were observed under bright- and dark-field conditions. Bar=1 mm.

hybrids compared with *L. minor* and MG hybrids, respectively. Furthermore, for Chl *b* and carotenoid content, significant differences were found between *L. minor* and the hybrid cytotype homoploid MG and in respect to *L. gibba* and the hybrid cytotype triploid GGM. *Lemna minor* (*L. minor* 5500 and *L. minor* 9482) had the highest pigment content. The estimated FW of a single frond of *L. gibba* is significantly different from that of *L. minor* and from both *L.* ×*mediterranea* cytotypes. No significant differences were found between *L. minor* and the homoploid cytotype MG. Equally high growth rates under the tested conditions were shown within and between the two species, *L. minor* and *L. gibba*, and the two hybrid cytotypes, MG and GGM. In particular, RGR in the period under study ranged from 0.17 g^{-1} d^{-1} to 0.25 g^{-1} d^{-1} for *L. gibba* and from 0.17 g^{-1} d^{-1} to 0.18 g^{-1} d^{-1} for GGM clones, while it ranged from 0.16 g^{-1} d^{-1} to 0.18 g^{-1} d^{-1} for *L. minor* and from 0.16 g^{-1} d^{-1} to 0.22 g^{-1} d^{-1} for MG. These RGRs values agree with data reported in the literature, which range from ~0.1 d^{-1} up to 0.3 d^{-1} (Zhang *et al.*, 2014; Van Echelpoel *et al.*, 2016). In the hybrid triploid cytotype, GGM, the protein content was lower and significantly different compared with the homoploid cytotype MG and the two parental species. Among the analysed accessions, *L. gibba* 9598 showed the highest values for single frond FW, RGR, and protein content.

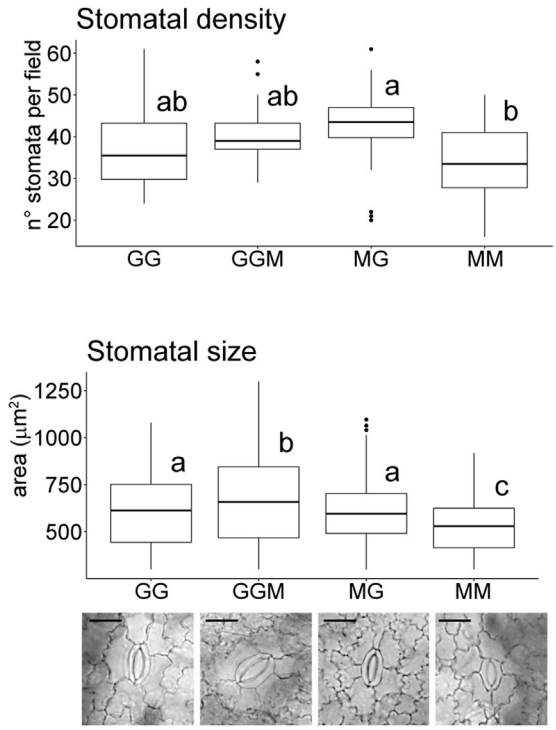

Fig. 10. Differences in stomatal traits between the two parental species, *L. minor* (MM) and *L. gibba* (GG), and the two *L. ×mediterranea* cytotypes (MG and GGM): stomatal density (above) and size (below). Box plots labelled with different letters indicate significant differences between grouped *Lemna* species and hybrid cytotypes (ANOVA followed by Tukey HSD, $P<0.05$). $n=3$. Representative examples of stomatal morphology (bottom) in each of the corresponding groups photographed by optical microscopy. Bars=30 µm.

Taxonomy

The taxonomic treatment, including the key morphological traits (Fig. 13) and the geographical distribution (Fig. 14), of the natural interspecific hybrid *L. ×mediterranea* and parental species (*L. minor* and *L. gibba*) is reported in Supplementary Fig. S5A.

Discussion

Multiple molecular data provided definite evidence that *L. minor* and *L. gibba* can spontaneously hybridize in nature, confirming previous data obtained by TBP analysis (Braglia *et al.*, 2021b). The new nototaxon, *L. ×mediterranea*, probably corresponds to the taxon described (not validly) as the new species '*L. symmeter*', discovered in Italy (Giuga, 1973; Supplementary Fig. S5B). Its hybrid nature was later hypothesized by Kandeler while reporting that the proposed taxon *L. symmeter* stands between *L. gibba* and *L. minor* and might even be a sterile hybrid between these two *Lemna* species (Kandeler, 1975). From the analysed samples, *L. ×mediterranea* is distributed over a wide geographic area, centred in the Mediterranean region.

The description of *L. ×mediterranea* underscores how in this hybrid there is the appearance of intermediate morphological traits between the two parental species, a common event in hybrids that often makes their morphological discrimination from the parental species challenging. Despite limited morphological differences between hybrids and parental species, morphometric analysis of several traits is in agreement with genetic analysis in supporting a clear distinction of *L. ×mediterranea* and also in separating the two cytotypes, homoploid (MG) and triploid (GGM), originating from both reciprocal crosses, as revealed by plastid marker analysis. Paradoxically, each of the two cytotypes is more similar to one of the parental species than to the reciprocal hybrid (Figs 7, 8). The first cytotype is more closely related to *L. minor* and the second to *L. gibba*. Whether this could be actually considered as a maternal effect or a gene dosage effect remains to be established.

The more distinctive morphological differences between the two cytotypes are mainly related to quantitative traits (frond width, frond area, length/width ratio, root length, aerenchyma extension, and vein number) and only secondarily to qualitative traits (hyaline frond edge and aerenchyma position). Analysis of stomatal morphological parameters (size and density) highlighted the presence of significant differences in guard cell size, which are the largest in the GGM cytotype (Fig. 10). This is likely to be related to its increased genome size, as already reported for Lemnaceae (Hoang *et al.*, 2019). Also for the ecophysiological traits observed, such as pigment content and RGR, hybrids have intermediate values, with triploid hybrids more closely related to *L. gibba* and diploid clones more similar to *L. minor*, suggesting that different genome contributions also affect biochemical traits and, possibly, plant physiological performance (Fig. 11). In conclusion, no morphological criterion is *per se* sufficient to provide unequivocal identification of *L. ×mediterranea* clones, and the use of molecular analysis is strongly suggested.

Population structure analysis inferred from AFLP data using different bioinformatics models supports the occurrence of different lineages, the parental populations, converging in the formation of an interspecific hybrid population. In this respect, the limited number of species-specific alleles detected in *L. ×mediterranea* suggests a fully and bipartisan genomic contribution of both parents merged in the hybrid. Phylogenetic network reconstruction also identifies the dual contribution of the parent species, placing the hybrid group closer to *L. gibba* than to *L. minor*, and supports its separation into at least two, possibly three, diverging clusters (Fig. 4), also in agreement with the similarity tree generated from TBP profiles (Supplementary Fig. S2). Greater similarity of hybrids to European clones of the parent species suggests their origin from a limited number of European ecotypes, in accordance with their lower intrataxon genetic diversity with respect to parental species. According to the NewHybrids results (Fig. 5), all clones have a high probability to represent F_1 populations. No evidence for backcrossing emerged for the seven clones, despite the fact that

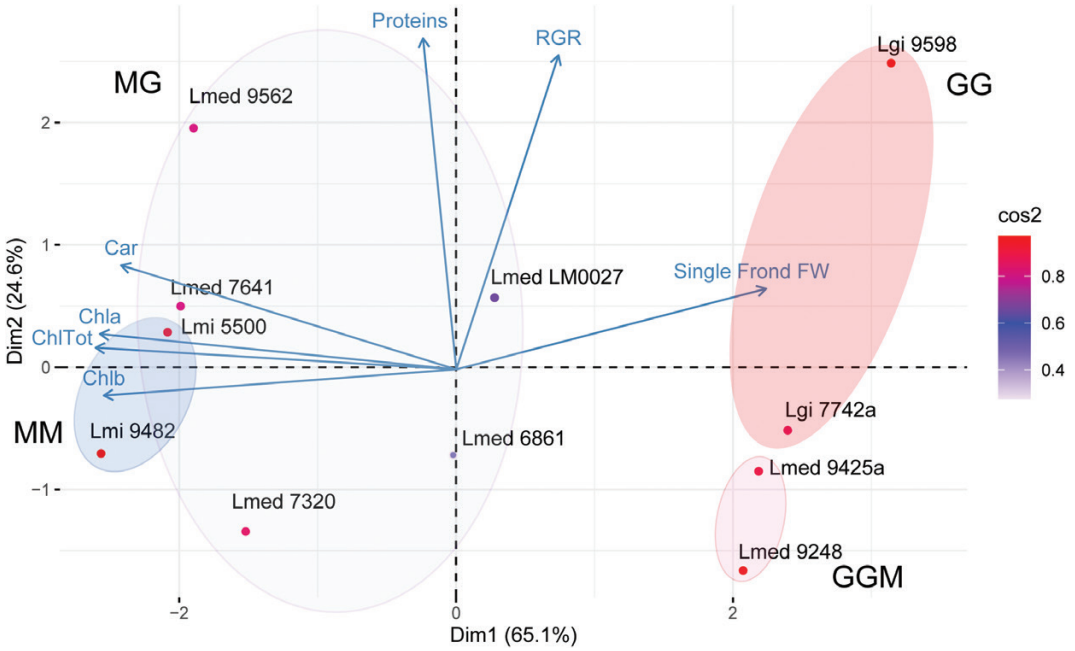

Fig. 11. Principal component analysis (PCA) of the measured growth data and biochemical parameters: relative growth rate (RGR), proteins, chlorophylls (ChlTot, Chla, and Chlb), carotenoids (Car), and single frond fresh weight (Single Frond FW) of the two parental species, *L. minor* (MM) and *L. gibba* (GG), and the two *L.* ×*mediterranea* hybrid cytotypes (MG and GGM). Plot for PC1 and PC2, where each oval encompasses the observed pattern of variance of each *Lemna* clone under the first two principal components clustering separately MM and MG (blue ovals) and GG and GGM (pink ovals), respectively.

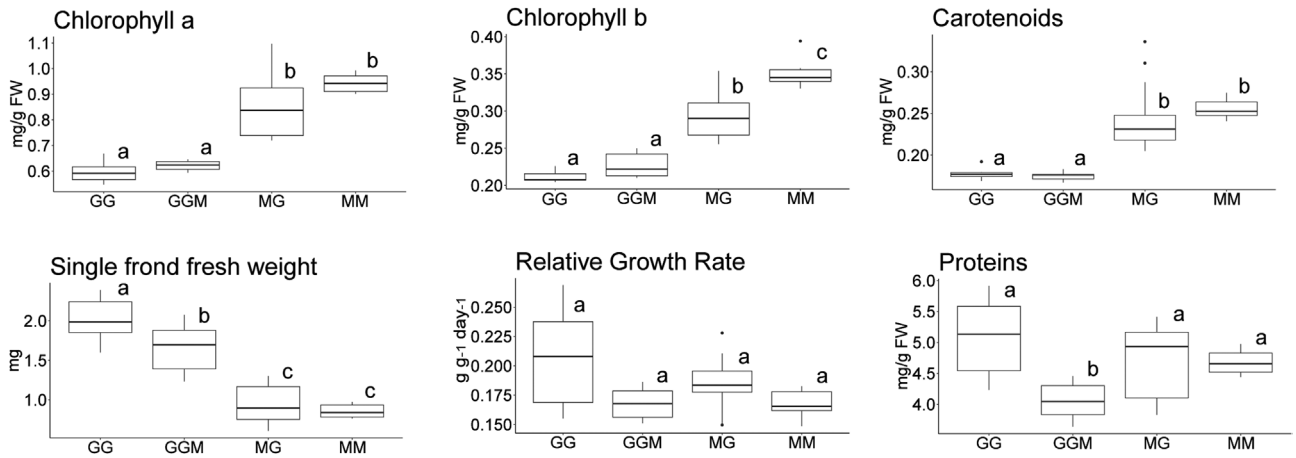

Fig. 12. Differences in photosynthetic pigment content, single frond FW, relative growth rate (RGR), and protein content between the two parental species, *Lemna minor* (MM) and *L. gibba* (GG), and the two *L.* ×*mediterranea* hybrid cytotypes MG and GGM. Box plots labelled with different letters indicate significant differences between different grouped *Lemna* species and hybrid cytotypes (ANOVA followed by Tukey HSD, *P*-value ≤0.05). *n*=5.

L. ×*mediterranea* may occur in association with either of the parental species, as reported for *L. symmeter* (Giuga, 1973) and for clones identified as non-gibbous forms of *L. gibba* but having the plastid haplotypes of *L. minor* (Marconi *et al.*, 2019). Such observations suggest sterility or very low fertility and self-incompatibility of hybrids. Flower induction experiments are ongoing to address these key points. Even very low rates of sexual reproduction are in fact considered sufficient to get rid of negative mutations that accumulate in asexual populations (Hojsgaard and Hörandl, 2015), making the establishment of self-evolving hybrid lineages possible, slowly leading to speciation. Although aggregates of vegetative reproducing individuals are unlikely to establish species-like lineages (Hörandl, 2022), in the case of homoploid hybrids, speciation is now accepted even if the hybrid lineage can be established as viable progenies through vegetative propagation, not necessarily requiring allopolyploidization (Comai, 2005; Sochor *et al.*, 2015; White *et al.*, 2018). In *Lemna*, hybrid population stability and diffusion can

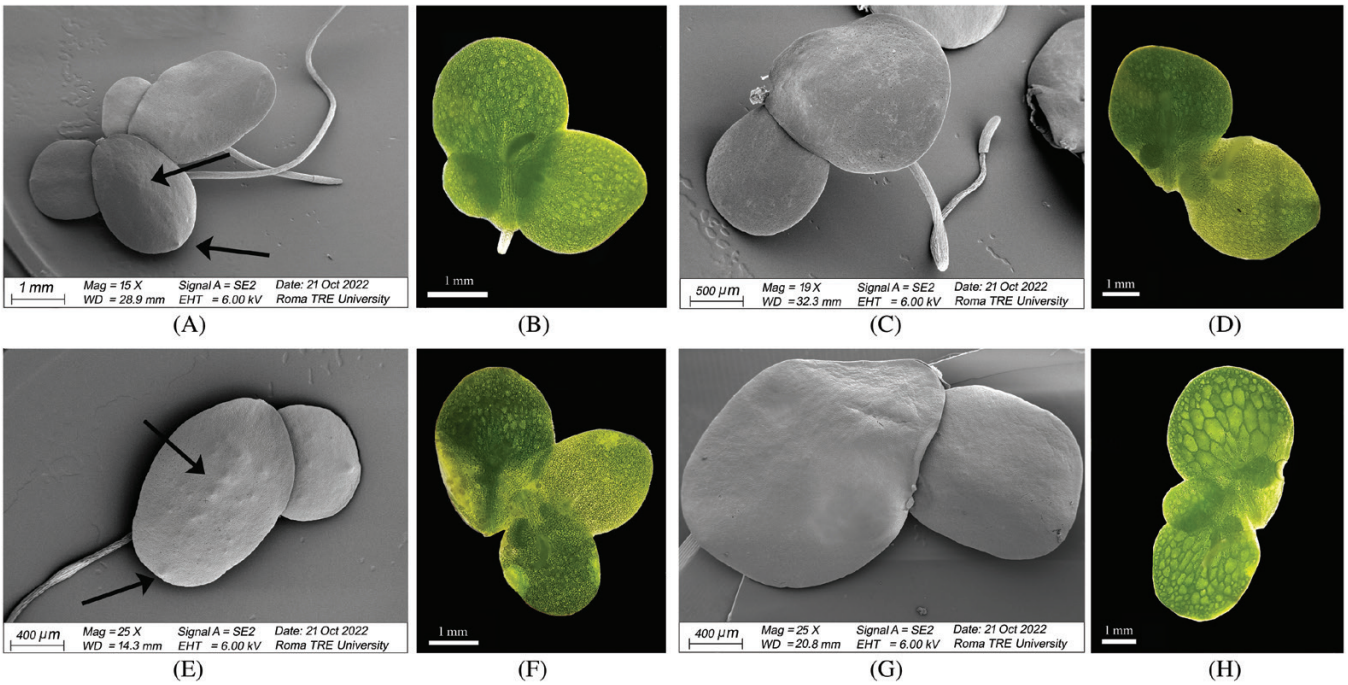

Fig. 13. Representative SEM and stereomicroscopic images. Representative images of the hybrids *L. ×mediterranea*—homoploid cytotype (MG), clone 9562 (A and B) *L. ×mediterranea*—triploid cytotype (GGM), clone 9425a (C and D) and the parental species, *L. minor* (E and F), *L. gibba* (G and H) at SEM (left) and stereoscopy (right). In detail: adaxial frond surface (A, C, E, G) and abaxial frond surface with visible aerenchyma (B, D, F, H). Dark arrows indicate serial or terminal papules on the adaxial frond surface (A, E).

be clearly provided by fast clonal propagation and long-distance dispersal of these tiny plants through water flow and zoochory (Coughlan *et al.*, 2017), promoting its establishment as a species if favoured by some competitive advantage with respect to the parental species. The success of *L. ×mediterranea* is evidenced by the large geographic area and collection dates of the hybrid clones from 1954 to 2011. Recovering living populations will provide further information on hybrid distribution and origin.

Another peculiarity of *L. ×mediterranea* is the presence of two different but unusual cytotypes, homoploids and triploids, while no tetraploid has been found to date. As both parental species are known to be mostly diploids (Landolt, 1986), the simplest explanation is that triploid hybrids originated from the fertilization of unreduced *L. gibba* ovules (2*n*) by normal haploid pollen cells (*n*) from *L. minor*. However, breeding between a tetraploid *L. gibba* and a diploid *L. minor* cannot be excluded. A somatic mutation leading to tetraploidy has been recently described for an *L. gibba* clone after long-term cultivation *in vitro* (Sarin *et al.*, 2023). Wide variations in both genome size and chromosome number have often been reported in *Lemna* and *Wolffia*, although not all old chromosome counting data are fully reliable (Hoang *et al.*, 2019, 2022). More recent data showed that triploid cytotypes are present in both *L. minor* and the hybrid species *L. ×japonica* that also includes homoploid hybrids (Ernst *et al.*, 2023), suggesting that hybridization and polyploidization are more common than previously thought in the *Lemanceae*.

Hybridization is extremely common in plants, and most successful hybrids are polyploid, a condition which grants full fertility eventually leading to hybrid speciation. Conversely, both homoploid and triploid hybrids are quite rare in terrestrial plants and are considered as bridges to form fully fertile, higher ploidy (tetraploid/hexaploid) species (Ramsey and Schemske 2002). In a very few cases, homoploid hybrids become stabilized over time, keeping an acceptable degree of fertility and becoming reproductively isolated from parents thanks to ecological or biological barriers (Mason and Pires, 2015) then becoming morpho-physiologically different, self-evolving species. Homoploid hybrid speciation (HHS) has been well documented in some plant species such as *Helianthus* (Schwarzbach and Reiseberg, 2002) or *Senecio* (Abbott *et al.*, 2013), but true numbers are likely to be underestimated (Yakimoski and Rieseberg, 2014). The number of known triploid plant species is even smaller, partially due to the triploid block effect, impairing endosperm development and inducing seed abortion (Köhler *et al.*, 2010). In such cases, at least at early stages, clonal propagation can provide an escape route to the low degree of fertility (Vallejo-Marin and Hiscok, 2016). An interesting example of a recently generated triploid species, *Cardamine ×insueta* Urbanska-Worytkiewicz, has been documented in the Swiss Alps (Urbanska and Landolt, 1972). The colonization of a new habitat provided almost completely reproductive isolation from the parental species while the acquisition of leaf vivipary enabled the hybrid to be a dominant

Fig. 14. Geographical origin of the seven *L.* ×*mediterranea* specimens. Circles indicate homoploid MG hybrids, triangles indicate triploid GGM clones.

species at the site despite its ploidy level (Sun *et al.*, 2020). More detailed analysis of ecological differences between *Lemna* hybrids and parental species is also needed to understand the advantages of hybrids and the possibility of their adaptation to different ecological niches even within the same water body.

This study further demonstrates that interspecific hybridization can be a common mechanism to generate diversity and variation in *Lemna*, which might have played an important role in the evolution and diversification of this genus and, possibly, in other genera of duckweeds. This is in accordance with findings by Les and Philbric (1993) who, analysing literature data for 117 genera of aquatic angiosperms, suggested that the high vagility (displacing ability) and rarity of sexual reproduction common to most of them have dramatically influenced the evolutionary consequences of two factors that have played major roles in the evolution of terrestrial angiosperms, namely hybridization and chromosome number change.

Supplementary data

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

Fig. S1. Image example used for measuring stomatal traits.

Fig. S2. Dendrogram generated by UPGMA clustering analysis of the TBP dataset.

Fig. S3. Nucleotide sequences gained by both the β-tubulin intronic regions of the locus *TUBB*1 and plastid markers.

Fig. S4. PCA of the AFLP dataset of *Lemna* clones analysed.

Fig. S5. (A) The taxonomic treatment of the natural interspecific hybrid *Lemna* ×*mediterranea* and parental species. (B) Giuga's booklet (Giuga, 1950).

Table S1. Oligonuclotide list.

Table S2. Chemico-physical composition of mineral water used as the aqueous medium for *Lemna* clone growth.

Table S3. List of the examined quantitative and qualitative morphological characters.

Table S4. Raw data from flow cytometry.

Table S5. Summary of the NewHybrids analysis for five random datasets consisting of 200 random AFLP loci each.

Table S6. *P*-value of ANOVA and χ^2 test for quantitative and qualitative traits, respectively.

Table S7. *P*-values of post-hoc analysis for quantitative traits significant from ANOVA.

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Author contributions

LM, SC, and MAI: conceptualization; MAI, GF, MF, SC, FM, EP, and LB: duckweed clone maintenance and investigation; MB, EP, MAI, GF, and LB: formal analysis; MM: data curation; SC, MAI, LM, and MB: writing—original draft preparation; LB, MB, SC, MAI, GF, and SG: writing—review and editing; MB, MAI, GF, MF, FG, SC, EP, MM, and LB: visualization; LM: supervision; SC, MAI, LB, and LM: funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Data availability

All data supporting the findings of this study are available within the paper. Plant material described in the manuscript is available for non-commercial research purposes upon request.

References

Abbott R, Albach D, Ansell S, *et al*. 2013. Hybridization and speciation. Journal of Evolutionary Biology **26**, 229–246.

Abbott RJ, Brennan AC, Hegarty MJ, Hiscock SJ. 2010. Homoploid hybrid speciation in action. Taxon **59**, 1375–1386.

Anderson EC, Thompson EA. 2002. A model-based method for identifying species hybrids using multilocus genetic data. Genetics **160**, 1217–1229.

Bog M, Appenroth KJ, Sree KS. 2019. Duckweed (Lemnaceae): its molecular taxonomy. Frontiers in Sustainable Food Systems **3**, 117.

Bog M, Appenroth KJ, Sree KS. 2020a. Key to the determination of taxa of Lemnaceae: an update. Nordic Journal of Botany **38**, doi:10.1111/njb.02658

Bog M, Lautenschlager U, Landrock MF, Landolt E, Fuchs F, Sree KS, Oberprieler C, Appenroth KJ. 2015. Genetic characterization and barcoding of taxa in the genera Landoltia and *Spirodela* (Lemnaceae) by three plastidic markers and amplified fragment length polymorphism (AFLP). Hydrobiologia **749**, 169–182.

Bog M, Sree KS, Fuchs J, Hoang PT, Schubert I, Kuever J, Appenroth KJ. 2020b. A taxonomic revision of *Lemna* sect. Uninerves (Lemnaceae). Taxon **69**, 56–66.

Borisjuk N, Chu P, Gutierrez R, Zhang H, Acosta K, Friesen N, Sree KS, Garcia C, Appenroth KJ, Lam E. 2015. Assessment, validation and deployment strategy of a two barcode protocols for facile genotyping of duckweed species. Plant Biology **17**, 42–49.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry **72**, 248–254.

Braglia L, Breviario D, Gianì S, Gavazzi F, De Gregori J, Morello L. 2021b. New insights into interspecific hybridization in *Lemna* L. Sect. Lemna (Lemnaceae Martinov). Plants**10**, 2767.

Braglia L, Gavazzi F, Gianì S, Morello L, Breviario D. 2023. Tubulin-based polymorphism (TBP) in plant genotyping. Methods in Molecular Biology **2638**, 387–401.

Braglia L, Lauria M, Appenroth KJ, Bog M, Breviario D, Grasso A, Gavazzi F, Morello L. 2021a. Duckweed species genotyping and interspecific hybrid discovery by tubulin-based polymorphism fingerprinting. Frontiers in Plant Science **12**, 625670.

Bretagnolle F, Thompson JD. 1995. Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. New Phytologist **129**, 1–22.

Ceschin S, Leacche I, Pascucci S, Abati S. 2016. Morphological study of *Lemna minuta* Kunth, an alien species often mistaken for the native *L. minor* L. (Araceae). Aquatic Botany **131**, 51–56.

Comai L. 2005. The advantages and disadvantages of being polyploid. Nature Reviews. Genetics **6**, 836–846.

Coughlan NE, Kelly TC, Davenport J, Jansen MAK. 2017. Up, up and away: bird-mediated ectozoochorous dispersal between aquatic environments. Freshwater Biology **62**, 631–648.

Da Silva HA, Scapim CA, Vivas JMS, do Amaral Júnior AT, Pinto RJB, Mourão KSM, Rossi RM, Baleroni AG. 2020. Effect of ploidy level on guard cell length and use of stomata to discard diploids among putative haploids in maize. Crop Science **60**, 1199–1209.

De Lange L, Pieterse AH. 1973. A comparative study of the morphology of *Lemna gibba* L. and *Lemna minor* L. Acta Botanica Neerlandica **22**, 510–517.

De Lange L, Pieterse AH, Van Baarsen-Beckers I. 1981. The occurrence of mixed populations of different genotypes of the *Lemna gibba*–*Lemna minor* complex. Acta Botanica Neerlandica **30**, 191–197.

De Lange L, Westinga E. 1979. The distinction between *Lemna gibba* L. and *Lemna minor* L. on the basis of vegetative characters. Acta Botanica Neerlandica **28**, 169–176.

Dray S, Dufour A-B. 2007. The ade4 package: implementing the duality diagram for ecologists. Journal of Statistical Software **22**, 1–20.

Earl DA, von Holdt BM. 2012. Structure Harvester: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources **4**, 359–361.

Ernst E, Abramson B, Acosta K, *et al*. 2023. The genomes and epigenomes of aquatic plants (Lemnaceae) promote triploid hybridization and clonal reproduction. bioRxiv doi:10.1101/2023.08.02.551673. [Preprint].

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology **14**, 2611–2620.

Giuga G. 1950. Su una presumibile proprietà caratteristica dei numeri primi. Istituto Lombardo di Scienze e Lettere. Rendiconto A **83**, 511–528.

Giuga G. 1973. Vita segreta di Lemnacee. *Lemna symmeter* G. Giuga—species nova. Napoli: Blario, 19.

Hammer O, Harper DAT, Ryan PD. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica **4**, 9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm. Accessed January 2024.

Hoang PTN, Fuchs J, Schubert V, Tran TBN, Schubert I. 2022. Chromosome numbers and genome sizes of all 36 duckweed species (Lemnaceae). Plants **11**, 2674.

Hoang PTN, Schubert V, Meister A, Fuchs J, Schubert I. 2019. Variation in genome size, cell and nucleus volume, chromosome number and rDNA loci among duckweeds. Scientific Reports **9**, 3234.

Hojsgaard D, Hörandl E. 2015. A little bit of sex matters for genome evolution in asexual plants. Frontiers in Plant Science **6**, 82.

Hörandl E. 2022. Novel approaches for species concepts and delimitation in polyploids and hybrids. Plants **11**, 204.

Huson DH, Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. Molecular Biology and Evolution **23**, 254–267.

Jakobsson M, Rosenberg NA. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics **23**, 1801–1806.

Kandeler R. 1975. Species delimitation in the genus *Lemna*. Aquatic Botany **1**, 365–376.

Kassambara A. 2023. Ggpubr package v. 3.4.2: Ggplot2 Based Publication Ready. Plots. https://rpkgs.datanovia.com/ggpubr/.

Kassambara A, Mundt F. 2020. Factoextra: extract and visualize the results of multivariate data analyses. R Package Version 1.0.7. http://www.sthda.com/english/rpkgs/factoextra

Köhler C, Mittelsten Scheid O, Erilova A. 2010. The impact of the triploid block on the origin and evolution of polyploid plants. Trends in Genetics **26**, 142–148.

Lämmler W, Bogner J. 2014. Elias Landolt and the duckweeds. Aroideana **37**, 81–88.

Landolt E. 1975. Morphological differentiation and geographical distribution of the *Lemna gibba–Lemna minor* group. Aquatic Botany **1**, 345–363.

Landolt E. 1980. Key to the determination of taxa within the family of Lemnaceae. Veröffentlichungen des Geobotanischen Institutes ETH, Stiftung Rübel **70**, 13–21.

Landolt E. 1986. The family of Lemnaceae: a monographic study. Vol. 2. Veröffentlichungen des Geobotanischen Institutes ETH, Stiftung Rübel **71**, 1–566.

Lefever S, Rihani A, Van der Meulen J, Pattyn P, Van Maerken T, Van Dorpe J, Hellemans J, Vandesompele J. 2019. Cost-effective and robust genotyping using double-mismatch allele-specific quantitative PCR. Scientific Reports **9**, 2150.

Les DH, Crawford DJ. 1999. Landoltia (Lemnaceae) a new genus of duckweeds. Novon **9**, 530–533.

Les DH, Crawford DJ, Landolt E, Gabel JD, Kimball RT. 2002. Phylogeny and systematics of Lemnaceae, the duckweed family. Systematic Botany **2**, 221–240.

Les DH, Philbrick CT. 1993. Studies of hybridization and chromosome number variation in aquatic angiosperms: evolutionary implications. Aquatic Botany **44**, 181–228.

Li S, Li L, Fan W, Ma S, Zhang C, Kim JC, Wang K, Russinova E, Zhu Y, Zhou Y. 2022. LeafNet: a tool for segmenting and quantifying stomata and pavement cells. The Plant Cell **34**, 1171–1188.

Lichtenthaler HK. 1987. Chlorophyll fluorescence signatures of leaves during the autumnal chlorophyll breakdown. Journal of Plant Physiology **131**, 101–110.

Marconi G, Landucci F, Rosellini D, Venanzoni R, Albertini E. 2019. DNA barcoding as a tool for early warning and monitoring alien duckweeds (*Lemna* sp.pl.): the case of Central Italy. Plant Biosystems **153**, 660–668.

Mason AS, Pires JC. 2015. Unreduced gametes: meiotic mishap or evolutionary mechanism? Trends in Genetics **31**, 5–10.

McGoey BV, Chau K, Dickinson TA. 2014. Stomata size in relation to ploidy level in North American hawthorns (*Crataegus*, Rosaceae). Madroño **61**, 177–193.

Nei M. 1973. Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences, USA **70**, 3321–3323.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences, USA **76**, 5269–5273.

Oksanen J, Blanchet FG, Friendly M, *et al*. 2020. vegan: Community Ecology Package. http://CRAN.R-project.org/package=vegan

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics **155**, 945–959.

Ramsey J, Schemske DW. 2002. Neopolyploidy in flowering plants. Annual Review of Ecology and Systematics **33**, 589–639.

R Core Team. 2022. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Sarin LP, Sree KS, Bóka K, Keresztes A, Fuchs J, Tyagi AK, Khurana JP, Appenroth KJ. 2023. Characterisation of a spontaneous mutant of *Lemna gibba* G3 (Lemnaceae). Plants **12**, 2525.

Schlüter PM, Harris SA. 2006. Analysis of multilocus fingerprinting data sets containing missing data. Molecular Ecology Notes **6**, 569–572.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods **9**, 671–675.

Schwarzbach AE, Rieseberg LH. 2002. Likely multiple origins of a diploid hybrid sunflower species. Molecular Ecology **11**, 1703–1715.

Sochor M, Vašut RJ, Sharbel TF, Trávníček B. 2015. How just a few makes a lot: speciation via reticulation and apomixis on example of European brambles (Rubus subgen. Rubus, Rosaceae). Molecular Phylogenetics and Evolution **89**, 13–27.

Sun J, Shimizu-Inatsugi R, Hofhuis H, Shimizu K, Hay A, Shimizu KK, Sese J. 2020. A recently formed triploid *Cardamine insueta* inherits leaf vivipary and submergence tolerance traits of parents. Frontiers in Genetics **11**, 567262.

Tang Y, Horikoshi M, Li W. 2016. ggfortify: unified interface to visualize statistical result of popular R packages. The R Journal **8**, 478–489.

Tayale A, Parisod C. 2013. Natural pathways to polyploidy in plants and consequences for genome reorganization. Cytogenetic and Genome Research **140**, 79–96.

Tippery NP, Les DH, Crawford DJ. 2015. Evaluation of phylogenetic relationships in Lemnaceae using nuclear ribosomal data. Plant Biology (Stuttgart, Germany) **17**, 50–58.

Ulrich I, Ulrich W. 1991. High-resolution flow cytometry of nuclear DNA in higher plants. Protoplasma **165**, 212–215.

Urbanska-Worytkiewicz K, Landolt E. 1972. Natürliche Bastarde zwischen Cardamine amara L. und *C. rivularis* Schur aus den Schweizer Alpen. Berichte des Geobotanischen Institutes der ETH Stiftung Rübel **41**, 88–100.

Vallejo-Marín M, Hiscock SJ. 2016. Hybridization and hybrid speciation under global change. New Phytologist **211**, 1170–1187.

Van Echelpoel W, Boets P, Goethals PL. 2016. Functional response (FR) and relative growth rate (RGR) do not show the known invasiveness of *Lemna minuta* (Kunth). PLoS One **11**, e0166132.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research **23**, 4407–4414.

Wang W, Wu Y, Yan Y, Ermakova M, Kerstetter R, Messing J. 2010. DNA barcoding of the Lemnaceae, a family of aquatic monocots. BMC Plant Biology **10**, 205.

White OW, Reyes-Betancort A, Chapman MA, Carine MA. 2018. Independent homoploid hybrid speciation events in the Macaronesian endemic genus *Argyranthemum*. Molecular Ecology **23**, 4856–4874.

Yakimowski SB, Rieseberg LH. 2014. The role of homoploid hybridization in evolution: a century of studies synthesizing genetics and ecology. American Journal of Botany **101**, 1247–1258.

Zhang K, Chen YP, Zhang TT, Zhao Y, Shen Y, Huang L, Gao X, Guo JS. 2014. The logistic growth of duckweed (*Lemna minor*) and kinetics of ammonium uptake. Environmental Technology **35**, 562–567.