A survey of duckweed species in southern Italy provided first occurrences of the hybrid *Lemna* × *mediterranea* in the wild

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Key terms

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Abstract

1. Interspecific hybridization and polyploidization are recognized as two main driving forces in plant evolution, shaping genomes and favoring evolutionary novelty and ecological adaptation. Recent studies have demonstrated hybridization within the genus *Lemna* (Lemnaceae Martinov). *Lemna minor* has given rise to two interspecific hybrids: *Lemna* × *japonica*, recognized as a species since 1980, and the newly discovered *L*. × *mediterranea*, identified among germplasm collection clones.
2. *L*. × *mediterranea*, a hybrid between *L*. *minor* and *L*. *gibba*, was hypothesized to correspond to the species *L*. *symmeter*, which was invalidly described about 50 years ago in Southern Italy.
3. A sampling campaign identified eight populations of the hybrid, at different sites across the Campania region, in Italy. The isolated specimens were found to be genetically identical by the nuclear marker Tubulin-based polymorphism (TBP), likely belonging to the same original clone (LER-LME) distinct from previously analyzed collection clones, suggesting recurrent hybridization. The natural hybrid clone is triploid, with *L*. *gibba* as the plastid donor. Morphology is very similar to *L*. *gibba*, although the typical gibbosity of this species becomes evident only upon flower induction. Flowers are protogynous and self-sterile.
4. Populations of both parent species, *L*. *minor* and *L*. *gibba*, were recovered during the survey, recording a high genetic variability in *L*. *minor.* Other Lemnaceae species, *Wolffia arrhiza* and *L*. *trisulca* were also occasionally present. The presence of the invasive species *L*. *minuta* seems to be less prevalent with respect to other Italian regions.
5. Synthesis: Five populations of the cryptic hybrid *L*. × *mediterranea* were discovered for the first time in the wild in Southern Italy. Clones isolated from these populations, sampled from distinct water bodies over an area of about 4200 Km^2, are genetically indistinguishable and likely originated from the same hybridization event. Thanks to high intron polymorphism, TBP provides a straightforward method for genetically identifying sterile clonal lineages and tracking their spatial and temporal distribution. Ecological factors including competition with parental and invasive species, niche and climate change adaptation, stability in time and space are to be investigated.

Introduction

Lemnaceae Martinov is a family of floating aquatic plants that populate lentic and slow-moving water bodies, thriving, particularly, in systems impacted by cultural eutrophication (Romano & Aronne, 2021; Romano *et al.*, 2022). The family accounts for 36 species, a number still subject to updating and revision, thanks to novel genetic and genomic approaches. Duckweeds are often addressed as the most miniature flowering plants (Romano *et al.*, 2022). Although most species produce fully functioning flowers, fruits, and tiny seeds, Lemnaceae tend to prefer vegetative propagation as a reproductive strategy. They are often considered to be the fastest-growing angiosperms (Ziegler *et al*., 2015) in fact, some species can double their biomass in less than two days (Romano *et al.*, 2024). Their exceptionally short lifespan, rapid asexual growth and straightforward in vivo conservation have made plants from the genus *Lemna* L. attractive as a model organism for fundamental ecological and evolutionary research (Laird and Barks, 2018; Acosta *et al.*, 2021). In the late nineteenth century, Darwin described how species adapted and evolved under the influence of natural selection. Research has thoroughly investigated the effect of natural selection on population genetics and evolution, but the matter is still far from a complete understanding (Rieseberg, 2001; Abbott & Brennan, 2014). The introduction of modern molecular techniques to population studies has been a critical factor in unravelling the natural adaptation of species to different environments and their interaction with one another (Rieseberg & Willis, 2007). An important opportunity for experimenting entirely new genetic combinations is represented by interspecific hybridization, as has been underlined numerous times in recent years (Wong *et al.*, 2022). This process can be defined as the gametic interaction across taxonomically distinct species, resulting in the formation of novel organisms that may eventually lead to hybrid speciation (Horandl, 2022). Although both plant geneticists and crop breeders have heavily recognized the importance of intra- and interspecific hybridization as tools for crop improvement and selection, species hybridization and introgression occur naturally and play a crucial role in plant adaptation to the natural environment (Rieseberg & Willis, 2007), and are essential parts of plant speciation (Rieseberg & Willis, 2007; Warner & Walworth, 2010). Plants of the Lemnaceae family occupy an important "ecological niche"; their floating nature sees them as a regulatory factor of the biodiversity co-existing in the water column underneath (Feller *et al.*, 2024). Thanks to their fast growth and adaptation to various sources and concentrations of nitrogen, their coexistence with other floating plants is a critical factor in what seems to be the solution of conquering the natural environment (Fang *et al.*, 2007; Feller *et al.,* 2024). The reproductive strategies of these tiny plants are so well fine-tuned that they pose an elevated risk of eutrophication in the wetlands (Feller *et al.*, 2024). In fact, duckweeds are also considered as biological indicators of eutrophication conditions. Thanks to their genetic characteristics and the increasing temperature of our planet, we must accustom ourselves to seeing eutrophicated waters by Lemnaceae plants (Peeters *et al.*, 2013). More so fast-growing alien species are spreading and, in some cases, competing with autochthonous ones (Ceschin *et al.*, 2016). Various research has shown that modelling their growth patterns is essential to estimate their growth and mitigate the effect on the natural environment (Peeters *et al.*, 2013; Feller *et al.*, 2024). Duckweed populations exhibit relatively high levels of genetic diversity despite their predominantly asexual reproductive strategy. Thanks to the combination of molecular markers and DNA barcoding, we can more precisely categorize these plants taxonomically, thus obviating the limited number of morphological traits. Genomic approaches make these plant amenable to biogeographical studies and cross-species comparative work, facilitating modern molecular ecology and evolutionary biology research.

As for other higher plants, interspecific hybridization also occurs within the Lemnaceae family as it has been recently demonstrated (Braglia *et al*., 2021a, b). The application of the ILP marker known as TBP (Tubulin Based Polymorphism), has been a critical factor in re-categorizing the taxon *Lemna japonica* Landolt, previously described as a species, as an interspecific hybrid within the family Lemnaceae (Braglia *et al*., 2021a). Another interspecific hybrid, between *L. minor* L. and *L. gibba* L.*,* referred to as *L.* ×

mediterranea Braglia et Morello, has been identified within the *in vivo* Lemnaceae germplasm collections (Braglia *et al.*, 2024). Due to the geographic origin of the investigated accessions, this hybrid was supposed to correspond to the putative species identified in the Campania region (Italy) and described in 1973 as *L. symmeter* Giuga, closely related to *L. gibba,* but differing from it for flower development and sterility (Giuga, 1973). The aims of this paper were: i) to retrieve wild populations of *L.* × *mediterranea* in the natural environment in the same region where *L. symmeter* was originally described, ii) to verify the possibility that the two taxa are the same iii) to provide information about distribution and biodiversity of the hybrid and its parent species. Interspecific hybrids, coming from plants endemic to a particular geographical area, are an unprecedented source of information. They adapt well, compete with parent as well as alien species, can be recollected at different time intervals, and are used as biomonitoring in studying the novel adaptation of plant species to a particular geographical area. Furthermore, sterile hybrids among facultative sexual species are also useful to investigate pure clonally propagating lineages, their mutation rate, lifespan, origin, and frequency. Thanks to their univocal vegetative reproduction, due to the sterile flowers, interspecific hybrids can help scientists to better understand the effect of induced environmental changes at a fast vegetative reproductive cycle (Mo *et al.*, 2022; Tao *et al.*, 2022; Du *et al.*, 2022).

Materials and methods

Plant Habitat Identification and In-Field Sampling

To investigate the presence in Italy of the nothotaxon *L.* × *mediterranea* and verify its possible identity to the putative species *L. symmeter*, as described by Giuga (Giuga, 1973), we have planned and conducted an extensive plant sampling campaign, in the Campania region. The field campaigns were conducted during June, July, and August of 2022. This period was chosen due to the presence of the optimal conditions for duckweed growth. More specifically, the presence of reduced water volumes and higher ambient temperatures help the proliferation of Lemnaceae plants.

To sample the plants in the natural environment, we have adopted a double approach, we gathered the historical data from Giuga's documentation and used satellite images (Google Earth Pro®). To validate the use of satellite images, we adopted a three-step process: 1) consultation of the waterways maps, 2) categorization of the waterways, and 3) exclusion of the waterways that did not match these two parameters. An additional parameter that was considered was the presence of green material in the waterways with the historical imagery tool of the Google Earth® software (https://earth.google.com/web). The waterways have been categorized into three main categories: Artificial Waterways (AW) category for all the manmade waterways (canals, wells and drainage channels); Natural waterways (NW) acronym was used for all the waterways represented in the category of streams, rivers, creeks, gullies, springs, or washes; Artificial Ponds (AP) category included all the wells, artificial lakes and water reservoirs (see Table 1). This integrative approach enhanced the precision of our habitat identification process.

Once the potential points were identified, we proceeded with the site visits. This was performed by creating driving maps and then visiting the precise pinned points either by car or by walking toward the selected site. During the field expedition we visited approximately 130 different sites (Figure S1). When the presence of duckweeds in the waterway was verified, we performed a thorough examination to ensure a comprehensive collection of a representative sample.

Waterways were not always accessible, so we employed a set of tools to sample plants from them. We specifically utilized telescopic nets for open water areas and buckets attached to long ropes for reaching otherwise inaccessible points. We collected samples manually in locations where direct access to the waterways was possible (Figure 1).

Figure 1. Different water bodies and collection strategies. Representative aquatic environments present in the Campania region: A) the historical site of Real Sito della Lanciolla (AW) (S6), B) Pagani city centre (AW) (S11), C) the Volturno river (NW) (S3), and D) a water well in the National Park of Cilento (AP) (S14) In the image we can see the author Leone Ermes Romano collecting Lemnaceae in the different water environments.

Each plant sample gathered during sampling was catalogued by the sampling group (Table 1). To each sampling group, georeferenced coordinates were assigned (Garmin Fenix 6 watch). Upon return to the laboratory, collected plants were rinsed with fresh water to remove any adhering debris or contaminants. Subsequently, the plants were carefully examined under a stereo microscope to assess their morphological traits. Although morphological trait analysis is not sufficient for reliably discriminating different species within the family Lemnaceae, we performed this analysis to separate the putatively different specimens collected at each site. Following the morphological analysis, individual fronds were carefully separated from the clusters for sub-culturing, to produce clonal progeny. Plant sub-culturing was performed in a controlled environment at 20°C, under 16/8 hours photoperiod in a Velp incubator. Upon reaching sufficient biomass in the sub-cultured material, surface sterilization according to Appenroth's protocol (2015) prepared the samples for subsequent axenic propagation and genetic analysis. A total of 42 Lemnaceae have been clonally propagated and are summarized in Table 1.

Table 1. Fieldwork sampling summary where the column Sampling group shows the different samples collected during the expeditions; the column Accession describes the accession number attributed to the different specimens first identified via morphological characteristics; the column TBP categorizes the species to which each accessions belongs; the column Type of waterways describes the different waterways: Artificial Waterways (AW), Natural waterways (NW), Artificial Ponds (AP); the column Coordinates indicates the geographical location of the different samplings sites.

Sampling Group	Accession	TBP	Type of waterway	Coordinates	
				N	E
S1	LER001	*Lemna minuta*	AW	40°57.3113'	14°01.7699'
S2	LER002	*Lemna gibba*	AW	40°58.1384'	14°01.4786'
S2	LER003	*L. minuta*	AW	40°58.1384'	14°01.4786'
S3	LER004	*Wolffia arrhiza*	AW	41°00.8751'	14°00.8985'
S3	LER005	*L. gibba*	AW	41°00.8751'	14°00.8985'
S3	LER006	*L. minuta*	AW	41°00.8751'	14°00.8985'
S3	LER007	*L. gibba*	AW	41°00.8751'	14°00.8985'
S3	LER008	*L. minuta*	AW	41°00.8751'	14°00.8985'
S3	LER009	*L. gibba*	AW	41°00.8751'	14°00.8985'
S4	LER010	*L. gibba*	NW	41°02.7979'	14°03.1177'
S4	LER011	*L. gibba*	NW	41°02.7979'	14°03.1177'
S5	LER012	*L. minor*	AW	41°00.0776'	14°17.3984'
S6	LER013	*L. minuta*	AW	41°00.0403'	14°20.1625'
S6	LER014	*Lemna × mediterranea*	AW	41°00.0403'	14°20.1625'
S7	LER015	*Lemna minor*	AW	40°58.3651'	14°27.4156'
S8	LER016	*L. gibba*	AW	41°00.021'	13°59.160'
S9	LER017	*L. minor*	AW	41°15.7129'	14°52.9815'
S10	LER018	*L. minor*	AW	41°00.481'	13°58.518'
S10	LER019	*W. arrhiza*	AW	41°00.481'	13°58.518'
S11	LER020	*L. × mediterranea*	AW	40°45.0092'	14°31.6233'
S11	LER021	*L. × mediterranea*	AW	40°45.0092'	14°31.6233'
S11	LER022	*L. × mediterranea*	AW	40°45.0092'	14°31.6233'
S11	LER023	*nd*	AW	40°45.0092'	14°31.6233'
S11	LER024	*W. arrhiza*	AW	40°45.0092'	14°31.6233'
S12	LER025	*L. × mediterranea*	AW	40°30.9748'	15°05.0356'
S12	LER026	*L. minor*	AW	40°30.9748'	15°05.0356'
S12	LER027	*L. × mediterranea*	AW	40°30.9748'	15°05.0356'
S13	LER028	*L. minor*	AW	40°30.2460'	15°05.5225'
S13	LER029	*L. minor*	AW	40°30.2460'	15°05.5225'
S14	LER030	*L. minor*	AP	40°25.8012'	15°13.0328'
S15	LER031	*L. minor*	NW	40°21.9662'	15°35.7301'
S16	LER032	*L. trisulca*	NW	40°21.3626'	15°36.8687'
S16	LER033	*L. minuta*	NW	40°21.3626'	15°36.8687'
S16	LER034	*L. minor*	NW	40°21.3626'	15°36.8687'
S17	LER035	*L. minuta*	NW	40°21.1460'	15°37.9503'
S17	LER036	*L. minor*	NW	40°21.1460'	15°37.9503'
S18	LER037	*L. minor*	NW	40°21.2062'	15°38.1312'
S19	LER038	*L. minor*	NW	41°26.2255'	14°13,2147'
S20	LER039	*L. × mediterranea*	NW	41°24.3377'	14°06,3202'
S21	LER040	*L. minuta*	NW	41°25.1195'	14°05,5152'
S21	LER041	*L. gibba*	NW	41°25.1195'	14°05,5152'
S21	LER042	*L. × mediterranea*	NW	41°25.1195'	14°05,5152'

DNA Extraction

DNA was extracted by grinding 50-100 mg of frozen fronds, in Eppendorf tubes with 3 steel beads and a few mg of quartz sand with a TissueLyser II (Qiagen) at 30 Hz for 90 sec. Plant tissue was lysed following the standard procedures of the DNeasy Plant Kit. DNA was eluted in a final volume of 100 uL of 5mM Tris-HCl and stored at -20°C.

TBP Amplification, Capillary Electrophoresis, and Data Analysis

TBP amplification was performed according to the protocol reported by Braglia *et al*. (2020). Fluorescence-labelled amplicons were separated by capillary electrophoresis on a 3500 Genetic Analyser (Thermo Scientific) as described by Braglia *et al*. (2023). Each sample DNA was independently analyzed twice. The amplicon sizing and allele detection of the TBP electropherogram gained by both intron regions (I and II) was performed by Gene Mapper Software v. 5.0 (Thermo Fisher Scientific, Germany). The peak size (base pairs) and height (RFUs) of each pherogram were collected through a Microsoft Office Excel file and all the TBP profiles were aligned according to the peak size. The peaks (markers) scoring was performed considering both intron regions and a single presence/absence matrix (1/0 respectively) was then generated. FAMD (Fingerprint Analysis with Missing Data) program, v.1.31 (Schlüter & Harris, 2006) was used to estimate genetic parameters: percentage of polymorphic markers, number of fixed markers, number of private alleles (only meaningful if groups are mutually exclusive) found in each group (LER-LGI, *L. gibba*; LER-LME, *L.* × *mediterranea*; LER-LMI, *L. minor*). Multivariate analyses were inferred using Jaccard's similarity index implemented in Past 4 software (v. 4.13) for Windows (Hammer *et al*., 2001) and UPGMA (Unweighted Pair Group Method with Arithmetic mean) trees and a principal component analysis (PCA) were constructed. The measure of how faithfully the designed dendrogram preserves the estimated genetic distances was evaluated through the Cophenetic correlation coefficient using the Past 4 software. The TBP presence/absence data matrix was used to estimate: the (dis-)similarity values by a pairwise comparison analysis - as the proportion of shared diversity to total diversity (Whittaker, 1972) within and between the three LER *Lemna* groups, according to Koleff *et al*. (2003); the Shannon's diversity index – as the measure of the clone richness and relative abundance - within the three species of interest and including in the analysis all the available clones belonging to the CNR - Institute of Agricultural Biology and Biotechnology Duckweed Collection (IBBA DW Collection https://biomemory.cnr.it/collections/CNR-IBBA-MIDW; Supplementary Table S1), using the same software.

Plastid marker analysis

The *atpF-atpH* spacer and *rps16* barcoding region were used as plastid sequence markers for *Lemna* sp. and *Wolffia* sp., respectively. Primers and PCR conditions were as reported by Braglia *et al*. (2021a) and Bog *et al*. (2013). Amplicons were purified with the Microclean kit (Labgene Scientific) and sent to the Microsynt facility for sequencing on both strands. After trimming and polishing, sequences were either aligned using the BioEdit alignment tool to identify SNPs or used as probes for BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Frond Area

Among the different clones collected for the experiment, we selected three (LER002, LER012 and LER014). as representative of the hybrid and its parental species, for frond comparison. More specifically, we expected *L.* × *mediterranea* to show significant differences in frond size when compared to the parent species *L. minor* and *L. gibba,* as reported by Braglia *et al*. (2021b, 2024), Plants cultivated under the same environmental parameters were imaged after 168h of growth. We have used an Olympus SZX9 stereo

microscope, equipped with a Sony Alpha II camera to image the plants. Thirty fronds per clone were measured utilizing Image J software as described by Romano *et al*. (2022).

Flower Induction

Lemna gibba (7742), *L.* × *mediterranea* (LER021) and *L. minor* (5500) colonies were grown on modified Hutner's medium (Hutner 1953) lacking NH_4^+ and supplemented with 1% sucrose under long-day conditions (16:8 light/dark cycle) at 25°C and 11,750 lux of fluorescent light, in presence of 30 µM salicylic acid (SA) to check fronds' growth. Fifteen *L.* × *mediterranea* fronds were fixed on 0.3% agar-added same medium above. The flower development was monitored through observations and photographs at time intervals of three to 15 hours, starting 11 days after flower-inducing culture initiation. For comparison, *L. minor* and *L. gibba* flower development was investigated, and images of final flower structure and pollen production were shown.

Results

Genetic identification of the isolated clones, geographic distribution, and plant associations

Upon visiting about 130 sites across an area of about 4200 km^2, populations of Lemnaceae were found at 21 sampling sites (Figure 2). As morphological identification in the Lemnaceae is no longer considered sufficient for species identification in most cases, all subcloned samples were genetically identified. The three *Wolffia* samples were easily identified by morphology at the genus level, but species assignment was confirmed by sequencing the *rps16* plastid marker. BLAST analysis of the three identical sequences obtained (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 06/24/2024) retrieved *Wolffia arrhiza* (L.) Horkel ex Wimm. clone 8272 (HE819982.1) as the highest scoring based on best match, with a 98.55% identity over 968 nucleotides. The three *W. arrhiza* specimens shared identical TBP fingerprinting profiles, suggesting they belong to the same clonal population, closely related to other collection clones from Italy but genetically more distant from other populations (manuscript in preparation).

Each subcloned *Lemna* specimen was genetically identified exclusively by the TBP marker, able to discriminate all closely related *Lemna* species and their hybrids (Braglia *et al*., 2021a, b).

Table 1 reports the identity of 41 duckweed clones (LER023 was lost before analysis), belonging to *W. arrhiza* and to four *Lemna* species: *L. gibba, L. minor, L. minuta* Kunth and *L. trisulca* L. In addition, we were also successful in retrieving the interspecific hybrid between the first two *Lemna* species, *L.* × *mediterranea*, so far reported only from *ex-situ* germplasm collections (Braglia *et al*., 2021b; 2024). The geographic distribution of the 21 sampling sites is reported in Figure 2. *Lemna minor* was the most common species, found in 12 out of 21 sites, followed by the alien species *L. minuta*, at seven sites, *L. gibba, and L.* × *mediterranea,* at five sites each, and *W. arrhiza* recovered from three sites. *Lemna trisulca* was found at one site only. The presence of a single species occurred at ten sites, while an association of two or three species were found at eleven sites.

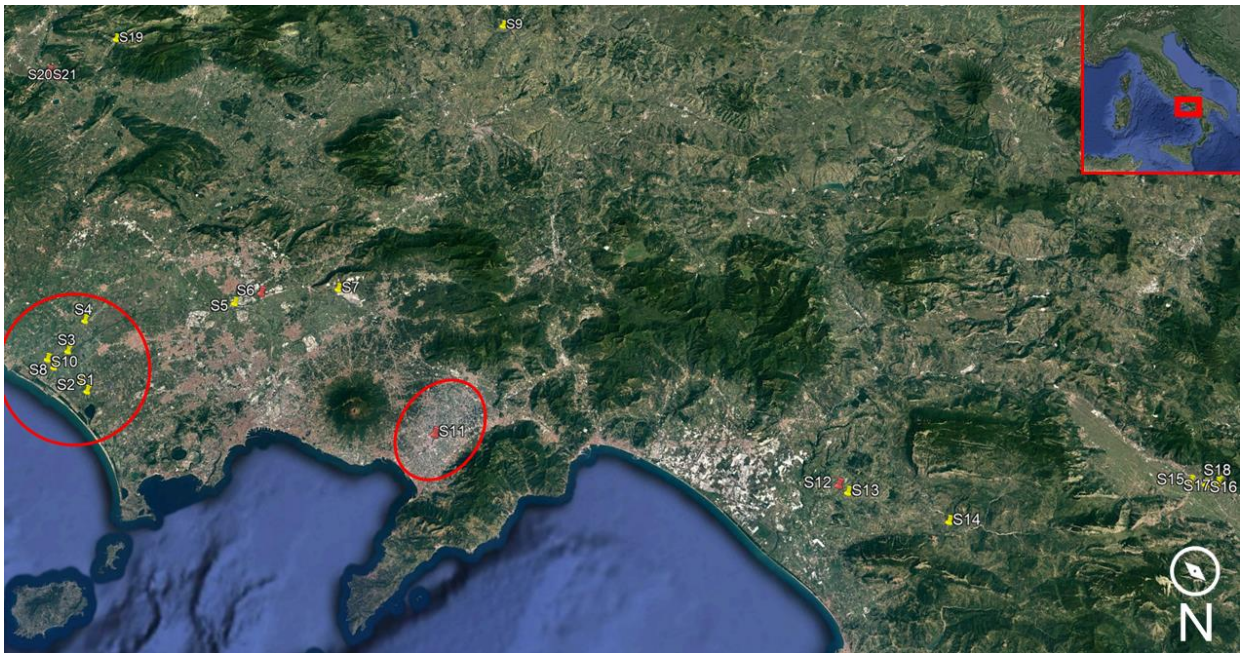

Figure 2. Map of the sampling campaign. Yellow pins indicate sampling sites and red pins indicate sampling sites in which there was also an occurrence of *Lemna* × *mediterranea* (see Table 1). Red circles show the sites in which *Lemna symmete*r was described. See supplementary material for the sampling map.

Characterization of *Lemna* × *mediterranea* clones

Lemna × *mediterranea* was identified in the wild for the first time in this work, in the Campania region, where *L. symmeter* was described as a new species (Giuga, 1973), and no more reported since then. Populations were found at five different sites (S6, S11, S12, S20 and S21) distributed over an area of at least 4200 km^2, alone or in association with either *L. minor* or *L. gibba*, but in no case with both parental species. One of the sites, S6, falls within one of the two main areas described for *L. symmeter.* Unfortunately, none of the plants observed and described by Giuga were preserved as herbarium specimens and no molecular comparison could be made.

All eight accessions, LER 014, 020-022, 025, 027, 039 and 042 showed identical TBP profiles for both the first and second tubulin introns, suggesting they have a clonal origin from a single hybridization event (see below). They will be collectively referred to as LER-LME.

Absolute genome size measurement of clone LER027 (758 Mbp) and comparison with previously investigated *L.* × *mediterranea* clones from the Landolt collection showed it was in the same size range of two accessions from South Tyrol (9248; 780 Mbp) and Northern Germany (9425a; 774 Mbp), respectively. These two clones were found to be triploid, with two *L. gibba* and one *L. minor* subgenome contributions, using a qPCR approach (Braglia *et al*., 2024). Other hybrids from the *ex-situ* collection were instead found to be homoploid. Sequencing of the *atpF-atpH* intergenic plastid region identified *L. gibba* as the maternal parent for the eight *L.* × *mediterranea* clones isolated in this work, as well as for the two collection clones mentioned before. Sequences were 100% identical to each other (not shown).

Frond morphology. Frond morphology showed closer similarity of hybrids to *L. gibba* than to *L. minor*, with a less elongated shape (Figure 3). According to Braglia *et al*. (2021b), morphological analysis revealed larger fronds in triploid hybrids when compared to the parental species. The comparison of frond areas

revealed significant differences among the different taxa ($p < 0.001$). Specifically, the frond areas were as follows: LER002 (*L. gibba)* had an area of 4.57 ± 1.68 mm², LER012 (*L. minor*) had an area of 5.19 ± 2.02 mm², and LER014 (*L.* × *mediterranea*) had an area of 7.46 ± 2.39 mm² (Figure 3).

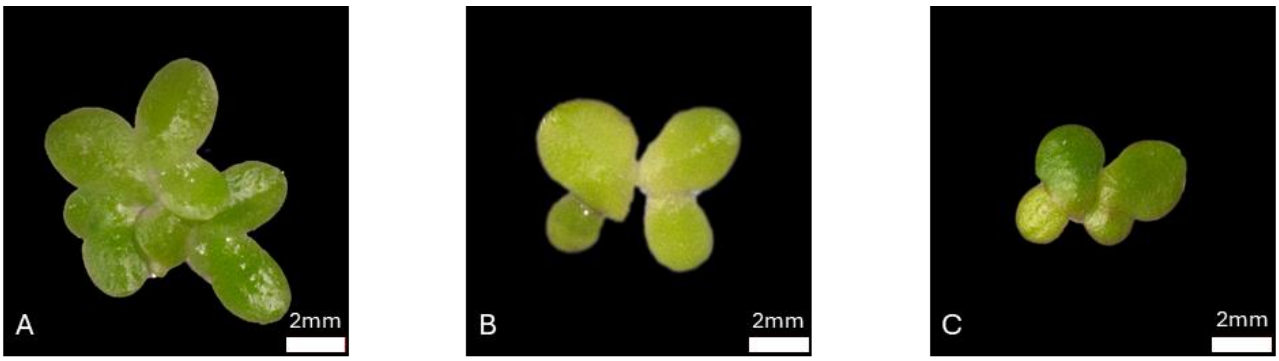

Figure 3. Representative images of frond clusters of the parental and hybrid taxa (from the left side to the right): a) LER012 (*L. minor*), b) LER014 (*L.* × *mediterranea*), and c) LER002 (*L. gibba).*

In addition, vein number was always five, like in *L. gibba*, when measured in fronds (Supplementary Figure 2) This number is typically three in *L. minor* and 3-4 in the diploid *L.* × *mediterranea* hybrid (Braglia *et al*., 2024).

Flower development.

One of the most distinctive traits mentioned as critical for *L. symmeter* species determination in Giuga's monograph was flower development. In *L. gibba* the first anther appears together or soon after the pistil, later followed by the second anther (Landolt, 1986). Protogyny was instead characterized in *L. symmeter* by the first appearance of the stigma, followed some days later by the simultaneous growth of both stamens together, when the pistil was already withered. We then induced flowering *in vitro* in one *L.* × *mediterranea* strain (LER021) in parallel with *L. minor* 5500 and *L. gibba* 7742a, for comparison.

A first effect, starting after about seven days of SA treatment, was the increase in frond thickness in *L. gibba*, conferring it the classical gibbose morphology that was not observed under normal laboratory cultivation conditions. The same effect was reported for EDDHA (De Lange and Pieterse, 1973). No such effect was seen in *L. minor,* while hybrids showed some volume increase of the aerenchyma, although less pronounced than in *L. gibba* (Figure 4).

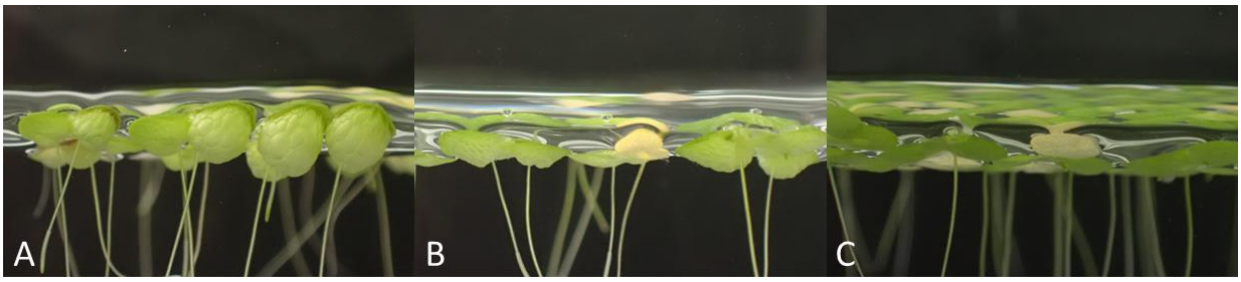

Figure 4. Frond morphology after salicylic acid (SA) addition. A. *Lemna gibba* (7742a); B. *L.* × *mediterranea* (LER021); C. *L. minor* (5500). The photos were taken 19 days after flower induction by SA. The hybrid *L.* × *mediterranea* has a visibly intermediate-sized aerenchyma between *L. gibba* and *L. minor* when they flower under *in vitro* conditions.

Salicylic acid treatment at 30 µM successfully induced flower formation in *L.* × *mediterranea* (LER021), observed as early as day 11 (Figure 5A). A total of 17 flowers were monitored for six days. Due to the short and irregular duration of some organs, their monitoring couldn't be strictly scheduled. Notably, the pistil emerged first, exhibiting prominent exudation on the stigma (Figure 5B). Following pistil maturation (Figure 5C), stamen growth is initiated, frequently appearing yellow or pale yellow and morphologically similar to fertile stamens. Despite the initial wilting of the first stamen, the pistil remained viable in most cases, albeit with a reduced level of stigma exudate (Figure 5D). Deviations from the pathway described were also observed. In some cases, the first stamen developed after the pistil completely withered, in other cases it did not develop even several days after the pistil had already withered. Following the first stamen wilting, the second stamen emerged from the frond pouch and apparently matured, but anthers did not burst despite the apparently normal flower development (Figures 5E and F). Anther dehiscence was detected in less than 50% of the scrutinized stamens, as seen in Figure 6, while it was clearly visible in the parent species (Figure 7) as it was also reported for other *Lemna* species (Fourounjian *et al*. 2021, Lee *et al*. 2024). Figure 7 illustrates the floral structure of the parental species, *L. minor* (5500) and *L. gibba* (7742a) in the same inducing conditions. Panels 7-A and 7-B depicted *L. minor*, while 7-C and 7-D showed *L. gibba*. including stamens and anthers, during pollen production. Notably, both species achieved complete anther maturation, evident by visible pollen release. As no fruit and seed set was observed in LER021, further investigations are needed to assess if this is due to self-incompatibility or, more likely, to altered pollen viability and/or ovule fertility.

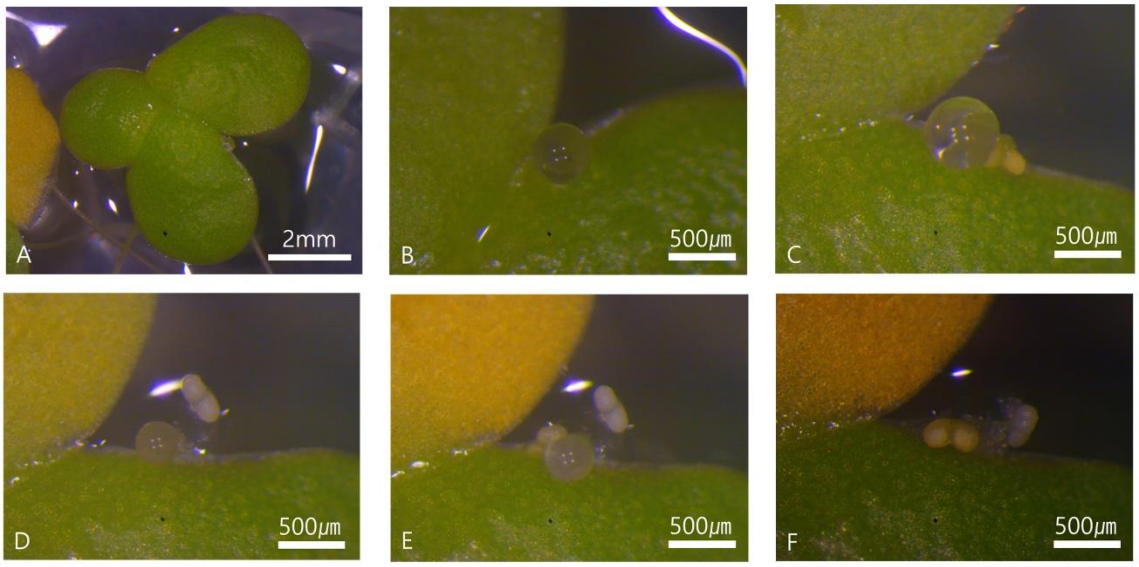

Figure 5. Flower development of *Lemna* × *mediterranea* (LER021). A) Whole flowering plant; B) Droplet on mature stigma; C) First stamen emergence and viable pistil; D) Pistil viability persistence despite wilting of the first stamen; E) Second stamen emergence; F) Fully developed second stamen following pistil and first stamen wilting.

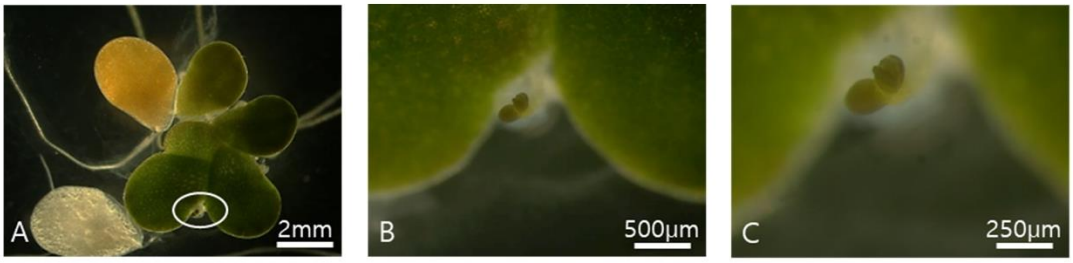

Figure 6. Flowering *Lemna* × *mediterranea* (LER021) plant A) Whole flowering plant; B) anthers opening for pollen release; C) magnification showing the vertical slit.

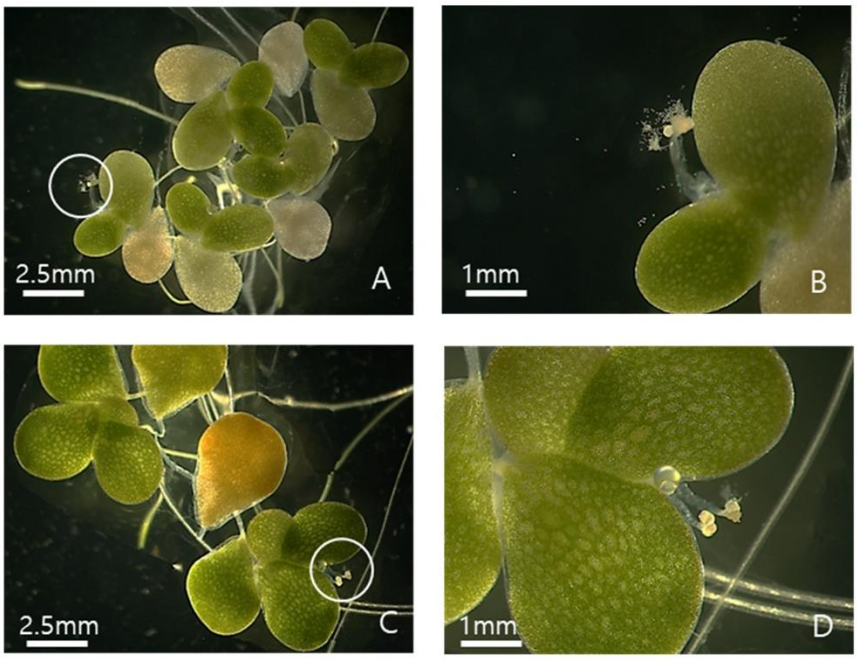

Figure 7. Flowers of *Lemna minor* (5500; A-B) and *L. gibba* (7742a; C-D). A-C) Whole flowering plants, B-D) Close-up view of flowers with dehiscent anthers releasing pollen.

Kinship and intraspecific genetic diversity within *Lemna* species

The peaks scoring of the TBP profile, limited to the *L. minor*, *L. gibba* and *L.* × *mediterranea* accessions (39), revealed 37 markers, all polymorphic, -21 and 16 from the I and II intron region, respectively. Although TBP investigates a limited number of loci and has lower resolution than other markers in the low-variability species *Spirodela polyrhiza* (L.) Schleid. (Bog *et al*., 2022a), it was able to score genetic diversity in our *L. minor* and *L. gibba* sample sets. When each species was considered separately, a different rate of intraspecific genetic variation was highlighted by the marker scoring: the highest allelic polymorphism was recorded in *L. minor* (15 polymorphic and 11 private markers); *L. gibba* accessions revealed only five polymorphic and three private markers, while no variation was recorded within *L.* × *mediterranea.*

Furthermore, the genetic relationships between accessions of the three *Lemna* taxa are shown in the UPGMA dendrogram in Figure 8, obtained by cluster analysis of the genetic similarity estimated on the TBP data (cophenetic correlation coefficient 0.9678). As the variability between duplicate analysis was zero (not shown) and there was a low risk of overestimating observed differences due to the limited resolution of the used marker, we considered each specimen as an independent clone when the Jaccard's coefficient of similarity was below 1, with respect to any other. As already mentioned, all *L.* × *mediterranea* specimens were then identical among each other and clustering with *L. gibba*, with which they shared more alleles than with *L. minor.* The eight *L.* × *mediterranea* clones in this study are then considered as a single clone and will be thereafter named LER-LME. By applying the same role, at least four different clones were found for *L. gibba* and 10 for *L. minor*. In this contest, the mean Jaccard's similarity estimated within the three taxa showed a higher value in *L. gibba* (0.8769) than in *L. minor* (0.6690), further highlighting a greater genetic variability within this latter.

Specimens belonging to the same *L. minor* clones (LER029, LER034, LER036 and LER037) were found at four different sites (S13, S16-S18), while at one site, S13, specimens representing two distinct clones were collected (Figure 8). The same was also true for *L. gibba* (sites S3, S4), despite the lower variability scored by the marker in this species.

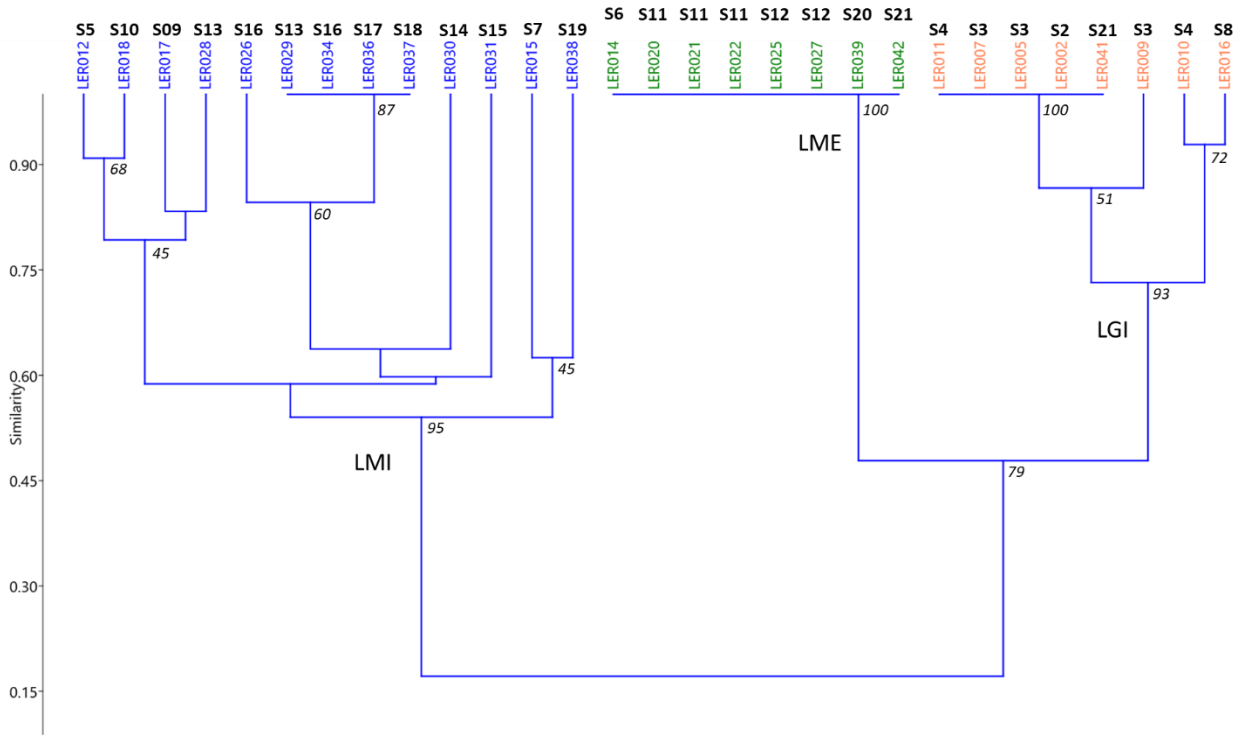

Figure 8. UPGMA dendrogram of the genetic distances among all collected LER-LME clones and those of the parent species, estimated on the TBP data. Sampling sites are indicated for each sample.

In addition, to identify putative parental clones of LER-LME, pairwise comparisons, according to the Whittaker formula, were estimated considering only those specimens classified as *L.* × *mediterranea* and its parental species (Supplementary Table S2). According to Table S2, identity between accessions becomes evident as much as the value approaches 1. Conversely, dissimilarity approaches zero when specimens are indistinct, and then considered as belonging to the same clone. As expected, the smallest mean value (0.00) was recorded comparing the eight *L.* × *mediterranea* specimens, while the highest value (1.00) was estimated between *L. minor and L. gibba* accessions. In this regard, single specimens from each LGI and LMI population could be identified as putative parental clones involved in the hybrid formation as those showing the lowest dissimilarity values with respect to LER-LME (Supplementary Table S3). When compared to *L.* × *mediterranea*, *L. minor* LER017 and *L. gibba* LER016 clones revealed the lowest dissimilarity values (0.39 and 0.28, respectively), and can be considered the most related to the hybrid. In agreement, the TBP pherogram comparison revealed a perfect allele overlap between these putative parental clones and the hybrid clones (Supplementary Figure S3).

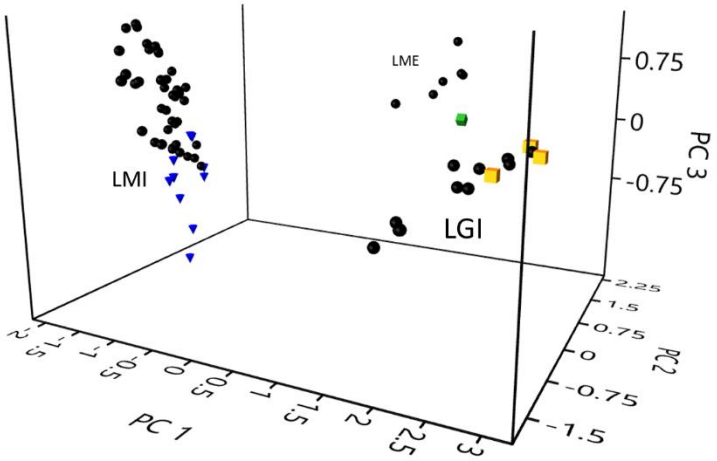

Figure 9. Principal component analysis (PCA) based on pairwise genetic distances calculated from the TBP matrices. Black dots represent clones of the three taxa belonging to the IBBA DW collection, green diamonds represent the LER-LME clone, blue triangles and yellow squares LER-LMI and LER-LGI respectively.

A 3D PCA was inferred considering all *L. minor, L. gibba* and *L.* × *mediterranea* clones included in the IBBA DW collection. Despite the limited number of considered TBP markers, 70% of the total variance was explained by the first three axes (48, 14 and 10% respectively, Figure 9) and all the analysed clones clustered into three distinct groups according to the three taxa. The hybrid clone LER-LME (green diamond in Figure 9) was clearly distinct from all other LME clones. LER-LMI (blue triangle in Figure 9) and LER-LGI (yellow squares in Figure 9) clones did not form separate clusters, although they were mostly concentrated at one border of the respective species distribution cloud. Concerning *L. minor*, the mean Shannon's diversity index, SI (mean ± SD) estimated within the group, the most densely populated PCA cluster, showed a non-significant difference comparing LER-LMI clones (SI = 2.92 ± 0.14) with all the clones of the IBBA DW collection (SI = 2.95 ± 0.13). This result remarks that, despite the limited number of analysed loci, the genetic diversity and the allelic richness characterizing the few collected clones of LER-LMI do not differ from those estimated for a more numerous and geographically widespread germplasm resource represented by clones included in the IBBA DW collection.

Discussion

This study provides the first direct finding of the interspecific hybrid *L.* × *mediterranea* in nature, unequivocally identified by molecular analysis. Eight specimens, collected at five distinct locations, could not be distinguished from each other either by the nuclear marker TBP or by the *atpF-atpH* plastid marker, and are assumed to belong to the same clone. Such triploid hybrid, fully sterile with high probability, as for the lack of seed setting upon flower induction, represents a precious way to trace the spread of purely clonal duckweed lineages over large areas over time. In fact, according to our survey, a single duckweed lineage can spread quite a long distance, over a 130 Km range, at least. As some of the five collection sites are not directly interconnected by water flow, frond transport through waterbirds by ecto- or endozoochory is the most likely mechanism for their spreading, through a step-by-step process (Coughlan *et al*., 2017a; Paolacci *et al*., 2023). Bird-mediated duckweed dispersal over short distances was demonstrated in controlled experimental settings (Coughlan *et al*., 2017b). By monitoring *L. minor* populations in Thuringia (N. Germany), identical distinct clones of *L. minor* were discovered in more than one pond, located at a distance of 1 km and 2.4 km from each other (Bog *et al*., 2022b). Anthropogenic activities are the second most probable way of propagule dispersal, particularly regarding cross-continental transport of alien species (Fedoniuk *et al*., 2022; Zielgler *et al*., 2023). Yearly sampling campaigns including more distant locations could provide interesting data about the stability and distribution of entirely clonal lineages of *L.* × *mediterranea*.

LER-LME is genetically different from all other *L.* × *mediterranea* clones so far identified within European duckweed germplasm collections (Braglia *et al*., 2021b; 2024). Four out of these seven clones were collected in Italy, at different places and times: clone 9248 was collected in 2000 in the Alpine region Trentino Alto-Adige, clones 9562 and 6861 came from in Central Italy, Trasimeno Lake (2016) and Massaciuccoli Lake (1954), respectively, while LM0027 was collected at the Botanical Garden of Naples, at an unknown date, in the same area of LER-LME. Except for 9248, the Italian clones were found to be genetically distinct homoploid hybrids, having *L. minor* as the female parent species, therefore surely independently originated from the LER clones in this study (Braglia *et al*., 2024). The triploid clone 9248, instead, although sharing with LER-LME the same maternal species, was the most geographically and genetically distant from the other clones in the PCA analysis (Figure 9).

At least five different hybridization events are then supposed to have originated the distinct clonal lineages so far recovered in Italy, suggesting that recurrent hybridization of *L. minor* and *L. gibba*, followed by clone dispersal is not an exceptional occurrence. Accumulation of somatic mutations over time, producing diverging clonal lineages from an original clone cannot be excluded but the very low mutation rate estimated for *L. minor* (Sandler *et al*., 2020) would imply very long stability of such lineages. Further investigation using higher-resolution markers and population structure analysis will help better understand ancestry and relationships between hybrid clones and their lifespan.

Previous indirect evidence for the presence of the cryptic hybrid in the Campania region comes from the description of sterile *Lemna* specimens resembling *L. gibba*, reported as the novel species *L. symmeter* (Giuga, 1973). Unfortunately, no specimens from the described populations are known to have been deposited in any herbaria at that time, so we could not evaluate its identity with any other *L.* × *mediterranea* clone. Flower development upon induction by SA in LER-LME differs from that reported for *L. symmeter*, characterized by the simultaneous development of the two stamens (from which the name symmeter). However, the non-physiological conditions of *in vitro* induction, in contrast with the naturally occurring flowering recorded by Giuga, could be responsible for the observed difference. Large variability of flower development among different clones of both *L. minor* and *L. gibba* was often observed *in vitro* (Landolt, 1980; Fu *et al*., 2017; Fourounjian *et al.*, 2021). Different maturation of sexual organs, protogyny and

homogamy, have been described even within the same species, *L. aequinoctialis* Welw., associated with self-sterility or fertility, respectively (Beppu *et al*., 1985; Lee *et al*., 2024). A further possibility is that differences among hybrid clones are associated with different genetic makeup, ploidy levels/subgenome composition/kind of cross, between our triploid clone and the populations described by Giuga. In accordance with *L. symmeter* description, we can quite surely affirm that LER-LME is at least self-sterile (possibly fully sterile), as it did not set fruits and seeds under the same induction conditions that were favourable for the parental species. From these and previous data, we then conclude that *L.* × *mediterranea* matches the description of *L. symmeter* and that the Italian peninsula represents an area favourable to *L.* × *mediterranea* formation and propagation. Further field studies may reveal additional hotspots for *L. gibba* and *L. minor* hybridisation. In this regard, interesting sites could be Northern Germany and the Netherland, where morphologically intermediate forms between *L. minor* and *L. gibba* were described in the past (De Lange and Pieterse, 1973; Landolt, 1975).

The two parental species *L. minor* and *L. gibba* were both largely present in the study area but, likely because of different ecological preferences (Landolt, 1987), never co-occurred in the same waterbody, suggesting this is not the most common occurrence. In agreement with recent reports (Bog *et al*., 2022b; Senevirathna *et al*., 2023; Schmid *et al*., 2024) *L. minor* was associated with high intraspecific genetic variability, scorable by the high polymorphic TBP marker: out of thirteen specimens, 10 have distinct allelic patterns. However, among all the isolated strains of *L. gibba* and *L. minor* we could find just two candidates as the putative parental clones, LER016 (*L. gibba*) and LER017 (*L. minor*) that showed the same allele combinations at β-tubulin loci found in LER-LME. Evidence for recurrent hybridization led us to suppose that flowering in *L. minor* is more frequent than often reported. Then, cross-pollination between the two species, when fronds are in close proximity, although unlikely, should give fertile seeds. Hybrids may locally have some competitive advantage over parent species allowing them to propagate and colonize new water bodies. Cross-pollination experiments are now ongoing to reproduce and test the success rate of interspecific hybridization *in vitro*.

Another piece of evidence in favour of underestimated crossing rates in the parent species comes from the high intraspecific variability highlighted in this study. Both parent species, particularly *L. minor*, showed intraspecific diversity between populations (sampling sites), as estimated by TBP. This agrees with recent population studies on *L. minor* worldwide, using different markers. Bog *et al*. (2022b) by using AFLP, found 20 distinct clones based on 36 samples collected in a small area in Thuringia, in the North of Germany, some of which living side by side in the same pond; Senevirathna *et al*. (2023) identified at least three distinct genetic clusters among 30 samples of *L. minor* from eight sites in Alberta (Canada) by using GBS, with sampling sites containing individual from the three clusters; by the same approach, Schmid *et al*. (2024) identified high inter-population diversity between 23 sampling sites across Switzerland, represented by eight distinct lineages. In accordance, in this study, we showed that *L. minor* populations can be polyclonal and that individual clones can be found at different sites over large distances.

The potential success of interspecific *Lemna* hybrids is clearly witnessed by *L. japonica* Landolt, described by E. Landolt as a species distinct from *L. minor* in 1980, native to East Asia (Landolt, 1980). Recent analysis revealed that this species is an interspecific hybrid between *L. minor* and *L. turionifera* Landolt (Braglia *et al*., 2021b; Ernst *et al*., 2023, preprint). In the wide original area, extending from Russia to China and including Japan and Korea, the two parent species are indeed sympatric. However, molecular analysis revealed that a large number of clones distributed across Asia and Europe and classified as *L. minor* belong instead to this cryptic taxon, despite the absence of the parent species *L. turionifera* in most of these areas (Braglia *et al*., 2021b; Volkova *et al*., 2023). Schmid *et al*. (2024) recently reported that *L. japonica* is widespread throughout Switzerland, by far distant from its putative centre of origin. As *L. japonica* has never been seen to set seeds, it is supposedly sterile as most interspecific hybrids. Its migration history must

solely rely on asexual propagation and long-distance spreading over time. Even in this case, recurrent hybridization is likely, as both homoploid and triploid clones with different subgenome compositions have been found among collection accessions (Ernst *et al*., 2023, preprint; Michael T., manuscript in preparation).

Other species found across the study area in Italy, included: *W*. *arrhiza*, *L*. *trisulca* (at one site only) and the alien species *L*. *minuta*. While *L*. *minor* and *L*. *gibba* are both autochthonous species commonly reported in Italy (Pignatti *et al*., 2017; Landolt 1986), *L*. *minuta* is an alien species, native to the American continent, but largely distributed in the Italian peninsula where it has been reported since 1989 (Desfayes, 1993). Since then, *L*. *minuta* has been widely reported in Northern and Central Italy, where it is described as naturalized or invasive in different regions (Iamonico *et al*., 2010; Iamonico 2012; Ceschin *et al*., 2018), in competition with *L*. *minor*. In Southern Italy, *L*. *minuta* stations were found in Apulia (Beccarisi & Ernandes, 2006), Sicily (Marrone & Naselli-Flores, 2011) and Calabria (Salerno & Ceschin 2015). The only record of *L*. *minuta* in Campania dates to 2017 and reported the species as casual (Stinca *et al*., 2017). Our data suggest that *L*. *minuta,* although spreading from North to South, didn't have the same character of invasiveness as in northern regions. The possibility of stronger competition by *L*. *gibba* and, possibly, *L*. × *mediterranea*, more common in the warmer Southern regions than in northern ones, is worth investigating.

No alien *Wolffia* species were reported in our survey. Although *W. arrhiza*, distributed in Europe and Africa, is the only autochthonous *Wolffia* species in Italy, other alien species are spreading to Europe from other countries. *W*. *columbiana* H. Karst. has been described in Europe and more recently also in Italy (Ardenghi *et al*., 2017) and *W. globosa* (Roxb.) Hartog & Plas has been expanding from Asia to Europe since 2010 (Kirjakov & Velichkova 2013).

Additional sampling campaigns, supported by molecular analysis, may reveal unknown aspects of *L*. × *mediterranea* distribution and the bases for their success.

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All authors have read and agreed to the published version of the manuscript.

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