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RESEARCH PAPER

Lemna aequinoctialis migrates further into temperate continental Europe—A new alien aquatic plant for Ukraine

Tetiana Fedoniuk[1] | **Manuela Bog**[2] | **Oleksandr Orlov**[3] | **Klaus Juergen Appenroth**[4]

[1]Polissia National University, Zhytomyr, Ukraine

[2]Institute of Botany and Landscape Ecology, University Greifswald, Greifswald, Germany

[3]Institute of Environment Geochemistry of National Academy of Sciences of Ukraine, Kyiv, Ukraine

[4]University of Jena, Matthias Schleiden Institute - Plant Physiology, Jena, Germany

Correspondence
Tetiana Fedoniuk and Klaus Juergen Appenroth, Staryy blvd., 7, Zhytomyr 10008, Ukraine.
Email: tanyavasiluk2015@gmail.com and klaus.appenroth@uni-jena.de

Abstract

The article presents the results of studies of a series of species identifications of the genus *Lemna* (Lemnaceae) conducted in 2019. Duckweed samples were taken from eight localities in the northern regions of Ukraine and identified to species using morphological and molecular markers (barcoding). A new alien species of duckweed for Ukraine—lesser duckweed (*Lemna aequinoctialis* Welw.)—was discovered, to which this study is devoted. All other samples belonged to *Lemna minor*. The article presents the most complete description of the distribution of *L. aequinoctialis* Welw. in Europe, and also contains updated data on the species distribution of duckweeds in Ukraine. Six species of the genus *Lemna* are now known in Ukraine—three native and three alien. The ecological requirements of *L. aequinoctialis* are similar to those of *L. minor*. We examined the morphological similarities and differences between these species, and confirmed the results by molecular barcoding using the plastid regions of the *atp*F-*atpH* and *psb*K-*psb*I intergenic spacers.

KEYWORDS
Araceae, barcoding, duckweed, Lemna, Lemnaceae, plant migration

1 | INTRODUCTION

The genus *Lemna* L. (Lemnaceae; Tippery et al., 2021) comprises 12 species worldwide (Bog, Appenroth, & Sree, 2020; Bog, Sree, et al., 2020). In the Checklist of vascular plants of Ukraine (Mosyakin & Fedoronchuk, 1999), four species of this genus were reported: *Lemna gibba* L., *Lemna minor* L., *Lemna trisulca* L., and *L. minuta* Kunth. The latter is alien (Ceschin et al., 2018), and was discovered in Ukraine for the first time in 1983 in the Danube Delta by Dubina and Protopopova (1983) (described as synonym *L. minuscula* Herter). In 2012, we discovered *Lemna turionifera* Landolt, another alien, invasive species, at three localities of northern Ukraine—Ukrainian Polesie (boreal Forest Zone) (Orlov & Yakushenko, 2013). In 2019, we found a new alien species of duckweed for Ukraine—*Lemna aequinoctialis* Welw., also called lesser duckweed, to which this study is devoted.

The invasion of *L. aequinoctialis* into temperate regions, including Europe, is evidently favored by global warming (Jacob et al., 2018). According to the Invasive Species Compendium (CABI, 2021; https://www.cabi.org/isc/datasheet/121132#tosummaryOfInvasiveness), *L. aequinoctialis* has been reported in Europe, Asia, Africa, North America, Central America, South America, the Caribbean, Australia, and Oceania (Figure 1) (for comparison see Landolt 1986: Li & Landolt, 2010). The

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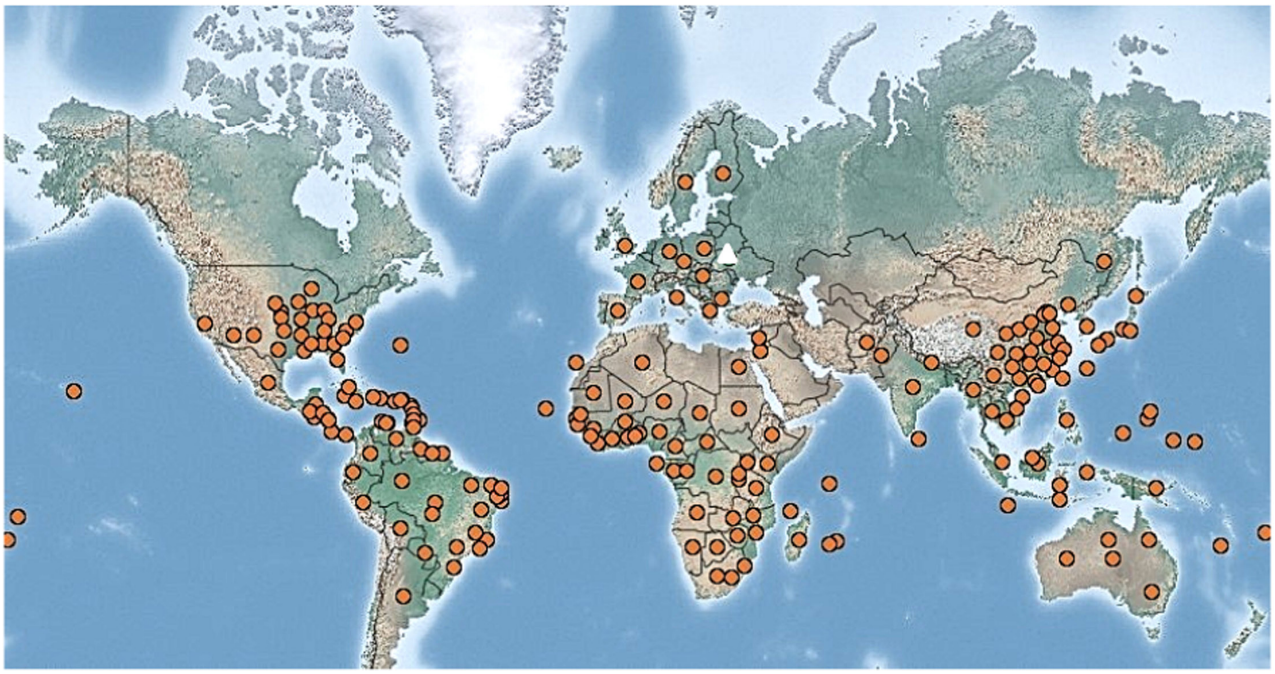


FIGURE 1 Worldwide distribution of *Lemna aequinoctialis* according to (https://www.cabi.org/isc/datasheet/121132#toDistributionMaps), modified. White triangle—Record in Ukraine.

distribution of *L. aequinoctialis* in Europe is dispersed, and in most European countries it is a rare alien species and most likely introduced accidentally into natural aquatic habitats (Toivonen, 1985). Landolt (1986) and Tutin et al. (1964) reported that in Europe *L. aequinoctialis* is locally naturalized in France, Germany, and Spain, and is frequent in rice fields in northern Italy. It was also introduced to Greece (http://www.plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:526159-1). Further records of *L. aequinoctialis* in Europe have been noted for Great Britain (https://bsbi.org/wp-content/uploads/dlm_uploads/Introduction-to-Duckweeds-Helena-Crouch.pdf), Sweden (Ryman and Anderberg 1999; Karlsson, 2003), Germany (Diekjobst, 1984), Italy (USDA-ARS, 2009), Spain (USDA-ARS, 2009), Austria (https://www.nobanis.org/national-species-info/?taxaid=6785&countryID=AT), Poland (Wolek, 1974), Hungary (Lukács et al., 2016), and Bulgaria (Kirjakov & Velichkova, 2016). In Russia, this species has not yet been identified in the European part, but it has been found in the Far Eastern Federal District (Soloveva & Lapirov, 2013).

For Ukraine, we report here the first record of *L. aequinoctialis*. Six species of the genus *Lemna* are now known in Ukraine—three native and three alien, including the invasive *L. aequinoctialis*.

2 | MATERIALS AND METHODS

Duckweed samples were collected from eight localities (Table 1), sterilized by treatment with the bleach Danklorix (4% of the bleach for 5 to 20 min) and tested for sterility thereafter in glucose-containing N-medium for 14 days (Appenroth, 2015). DNA was isolated from 15 mg silica gel-dried tissue using the CTAB method (Doyle & Dickson, 1987; Doyle & Doyle, 1987) and quantified spectrophotometrically at 260 nm. The plastidic regions of the *atpF-atpH* and *psbK-psbI* intergenic spacers, which were proposed as DNA barcoding regions for duckweeds (Borisjuk et al., 2015), were amplified in a total PCR reaction volume of 10 μl containing one-fold buffer B, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer (Wang et al., 2010), 0.05 U/μl hot start Taq polymerase (all reagents from Axon Labortechnik GmbH, Kaiserslautern, Germany), and 75 ng of genomic DNA. Amplification was carried out at 95°C for 12 min, followed by 35 cycles at 95°C for 45 s, at 55°C for 45 s, at 72°C for 45 s, and a final extension step at 72°C for 10 min. Purification of the PCR products was done using a 1:5 enzyme mix of Exonuclease I (20 U/μl) and Alkaline Phosphatase (1 U/μl) (both Thermo Fisher Scientific Inc., Waltham, MA, USA). Subsequently, Sanger sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). The cycle sequencing product was precipitated with ethanol, and loaded on a 3130 capillary Genetic Analyzer (Applied Biosystems). The query sequences were compared with sequences from a reference database for all duckweed species of one of the authors (MB).

Relative growth rates (RGRs) were measured on the basis of frond numbers as described previously (Naumann et al., 2007) using N medium (Appenroth

TABLE 1 Clones of the genus *Lemna* with origin in Ukraine

Clone ID	Coordinates	Locality	Date	Ecological characteristics
KJA018	50.238179, 28.831814	Zhytomyr region, Zhytomyr distr., Levkiv village, running water near the left bank of the river Teteriv	20.04.2019	Open place with full lighting, water inflow very slow, water eutrophic, depth 20–30 cm, bottom muddy, Lemna created solid carpet on the water surface
KJA019	50.238292, 28.832198	Zhytomyr region, Zhytomyr distr., Levkiv village, running water near the left bank of the river Teteriv	20.04.2019	Open place with full lighting, water inflow very slow, water eutrophic, depth 20–30 cm, bottom muddy, Lemna created solid carpet on the water surface
KJA022	51.088412, 28.405799	Zhytomyr region, Luhyny distr., Luhyny town, standing water near the river Zherev	30.09.2020	Semi-shadow place near trees, water standing, eutrophic, depth 20–30 cm, bottom oozy, Lemna created solid carpet on the water surface
KJA036	51.088335, 28.405960	Zhytomyr region, Luhyny distr., Luhyny town, standing water of oxbow of the right inflow near the river Zherev	30.09.2020	Semi-shadow place near trees, water standing, eutrophic, depth 20–30 cm, bottom oozy, Lemna created solid carpet on the water surface
KJA037	50.570673, 28.637909	Zhytomyr region, Horoshiv district, 1.3 km to the South from village Toporishche, drainage channel	09.11.2020	Open place with full lighting, water inflow very slow, water eutrophic, depth 10–20 cm, bottom sandy-oozy, Lemna created solid carpet on the water surface
KJA038	50.209153, 28.642368	Zhytomyr region, Zhytomyr distr., 0.5 km to the South from village Huyva, river Huyva, near the left bank	05.11.2020	Open place with full lighting, water inflow slow, water eutrophic, depth 20–50 cm, bottom oozy, Lemna created solid carpet on the water surface
KJA039	50.243872, 28.659941	Zhytomyr region, Zhytomyr distr., Zhytomyr sity, Teteriv river, near the right bank	25/09.2020	Open place with full lighting, water inflow slow, water eutrophic, depth 20–50 cm, bottom oozy, Lemna located in thickets of Glyceria maxima and created solid carpet on the water surface
KJA040	50.404243, 28.247260	Zhytomyr region, Zhytomyr distr., to the West from the Zhytomyr sity,, reservoir near the roadside Zhytomyr—Novohrad-Volynskyi	25.09.2020	Semi-shadow place near trees, water standing, eutrophic, depth 20–30 cm, bottom oozy, Lemna created solid carpet on the water surface

Note: Clone numbers were given according to international rules (Lam et al. 2020) together with the time of collection and some ecological characteristics.

et al., 2021) at 25°C and 100 μmol m^{-2} s^{-1} continuous white light for 7 days. RGR (d^{-1}), doubling time (DT; d), and relative yield (RY; g g^{-1} $week^{-1}$) were calculated as given by Ziegler et al. (2015). RY was defined as the number of fronds produced after 1 week starting with one frond (Ziegler et al., 2015).

3 | RESULTS AND DISCUSSION

All clones were first investigated for morphological markers using the identification key by Bog, Appenroth, and Sree (2020). Most clones were identified provisionally as *L. minor* but for two clones these markers indicated rather *L. aequinoctialis*. The most important similarities and differences of *L. minor* and *L. aequinoctialis* are summarized in Table 2. As *L. aequinoctialis* normally occurs in subtropical and tropical areas, this result was unexpected for Ukraine. Therefore, we applied the two molecular markers *atpF-atpH* and *psbK-psbI*, intergenic spacers analyzed after PCR amplification by sequencing for barcoding. The identity of the two clones, KJA018 and KJA019, was confirmed as *L. aequinoctialis*. However, these two plastid markers cannot distinguish between *L. minor* and *L. japonica* because the latter is most probably a natural hybrid between *L. minor* as mother and *L. turionifera* as the other parent (Braglia et al., 2021). The remaining clones, KJA022, KJA036, KJA037, KJA038, KJA039, and KJA040, were therefore identified provisionally by the molecular markers as *L. minor*/*L. japonica*. However, these two species can be distinguished by morphological markers (Bog, Appenroth, & Sree, 2020). According to our experience, two morphological markers are especially useful. In *L. minor*, the papula near the tip is not very distinct, and the fronds are mostly three-veined. If 4–5 veins are present, the outer lateral originates from the lower part of the inner lateral veins. In *L. japonica*, the papula near the tip is larger than the proximal one, and the 4th and 5th veins originate from the lower part of the inner veins, but not from the node. Using these morphological markers all

TABLE 2 Similarity and differences between *Lemna aequinoctialis* and *Lemna minor*

Organs	*Lemna aequinoctialis*	*Lemna minor*
Similarity		
Fronds	1–8 fronds are coherent in colonies	
	Linear dimensions overlap to a great extent	
	Surface flat	
	Three-veined, central vein nearly reaching apex	
	Mostly distinct papillae on upper surface near apex and above node	
	Turions absent	
	Margin entire	
Root	One	
Flower	Ovary with one ovule	
Seed in fruit	One	
Differences		
Fronds	More or less asymmetric	Symmetric
	Obovate to lanceolate	Obovate to elliptic
	Glossy green	Green, occasionally with reddish color on upper and/or lower surfaces
Root	Length 5–30 mm	Length 5–150 mm
	Sheath winged	Sheath not winged
	Apex sharp pointed	Apex rounded
Flowers	Opening—one side	Opening—at apex
	Stamen—2	Stamen—1
Fruit	Not winged	Winged laterally toward apex
	Asymmetric	Symmetric
Seeds	Falling	Staying
	With 8–24 distinct longitudinal ribs	With 10–16 distinct ribs

Note: In addition to the reference Bog et al. (Bog, Appenroth, & Sree, 2020; Bog, Sree, et al., 2020), the following reference was consulted: Azer (2013).

clones, identified by molecular barcoding as *L. minor*/*L. japonica* were identified as *L. minor*. Whereas *L. minor* is common in Ukraine, this is the first record of *L. aequinoctialis* in this country.

Lemna aequinoctialis was described for the first time by Welwitsch (1859): "Apontamentos Phytogeographicos 578. 1858 (1859) (December, 1859)" (Apont); locality: "Ella cresce abundantemente em quasi todas as laguas ao longo da costa, e nas aguas estagnadas á borda dos rios Quicombo, Loge, Lifune e Bengo; distribution: Angola." Lectotype: Angola, Distr. Luanda, 1858. F.Welwitsch 206 (*STU*). Synonims: *Lemna angolensis* Hegelm., *L. paucicostata* Engelm.," which translates as "It grows abundantly in almost all the lakes along the coast and in the stagnant waters on the banks of the Quicombo, Loge, Lifune and Bengo rivers; Distribution: Angola."

Landolt (1986) characterized *L. aequinoctialis* as an aquatic herb with a cosmopolitan tropical and subtropical distribution. Sometime later, it was confirmed that *L. aequinoctialis* is almost entirely restricted to warmer regions of both hemispheres (Crawford et al., 2006). Its seeds lack a dormancy state and are released from the fruit and germinate as soon as they are mature. The resulting fronds are not adapted to cold climates and therefore can be killed by the onset of freezing temperatures (Crawford et al., 2006).

All living samples of *L. aequinoctialis* and *L. minor* collected in Ukraine in 2019 were stored in the Institute of Botany and Landscape Ecology, University of Greifswald, Germany. This record of *L. aequinoctialis* is the most eastern known in Europe hitherto. We observed that this species successfully overwintered 2019–2020 and occurred in two plant communities (Table 3). In the first, the species population formed narrow long strips, with a width of 0.5–1.5 m on open water with low flow speed (0.4–0.5 $m \cdot s^{-1}$), in front of communities of riparian tall-grass vegetation. Water depth was 1–1.3 m, bottom muddy, with sludge layer thickness of about 30 cm. Projective cover of above-water layer was 95%–98% with the dominance of *L. aequinoctialis*: *L. aequinoctialis*—90–95%, *Spirodela polyrhiza* (L.) Schleid.—3%–5%. The under-water layer had a projected cover of about 20%, it consisted of *Ceratophyllum demersum* L.—10%–15% and *Lemna trisulca* L.—3%–5%. This community belongs to the alliance *Lemnion minoris*, order *Lemnetalia minoris* (Bolōs & Masclans, 1955), and class *Lemnetea* O. (Dubyna et al., 2019). Since in the described community all species, except *L. aequinoctialis*, were represented by common aquatic species of temperate latitudes, we attributed it to this alliance, although for the tropics of South America (Landolt, 1999) the order *Lemnetalia aequinoctialis* (Schwabe-Braun & Tüxen, 1981; Navarro & de Mera, 1992) was reported, which is characterized by more thermophilic species.

Additionally, *L. aequinoctialis* occurred in a riparian tall-grass community. The water depth was 40–50 cm, bottom muddy, with sludge layer thickness of about 30 cm. Total projective cover reached 70%–80%. The first layer with height 1.3–1.5 m consisted of some codominant species—*Glyceria maxima* (C.Hartm.) Holmberg (45%–50%) and *Typha latifolia* L. (20%–25%). *Bidens frondosa* L. (1%–3%) also occurred in this layer. The second layer with height 0.5–0.8 m was represented by *Sparganium erectum* L. (3%–5%), *Rumex hydrolapathum* Huds. – 1%, *Persicaria hydropiper* (L.) Delarbre (3%–5%), *Lythrum salicaria* L.—1%, and *Myosotis palustris* L.—1%. The third layer was represented by communities of free-floating *Lemnaceae* species, consisting of *L. aequinoctialis* (5%–10%) and *S. polyrhiza* (1%–3%). It belongs to the association *Glycerietum maximae* Nowinski 1930, corr. Šumberová, Chytrý et Danihelka in Chytrý 2011, alliance *Phragmition communis* Koch 1926, order *Phragmitetalia* Koch 1926, and class *Phragmito-Magnocaricetea* Klika in Klika et Novák 1941 (Dubyna et al., 2019).

Lemna aequinoctialis also created transitory dense mats with an area of 1–4 m^2 in front of obstacles on the water surface—tree trunks, twigs, or areas covered by reeds.

Probable sources of *L. aequinoctialis* dispersal in Ukraine into natural water ecosystems are as follows: (1) aquarium culture released in nature; (2) cultivation in greenhouses and basins with accidental release into natural biotopes; such dispersals are considered as sub-spontaneous; (3) middle-range drift of duckweed propagules from neighboring countries (Poland, Hungary, etc.) and also their more distant drift—from Southern Europe, Near East, North and Central Africa by waterfowl. The latter regions are important for wintering birds, and one of the most important bird migration routes in Ukraine runs along the river Teteriv. Three main modes of dispersal of duckweeds should be considered: hydrochory, exozoochory, and anthropochory. Hydrochory is the most important dispersal mode because frond abscission is very common for colonies of duckweeds (Henke et al., 2011), and small fragments (colonies of 2–3 connected fronds) will likely be dispersed by water flow within hydrologically connected systems (Coughlan et al., 2017). Also duckweeds are able to disperse rather easily by way of exozoochory—fronds which adhere to the legs and feathers of ducks and other waterfowl (Coughlan et al., 2015, 2017; Les et al., 2003). These birds are known to travel long distances even between continents. Probably, it can cause a long-distance transfer drift of duckweeds propagules from tropical and subtropical regions to temperate ones. Fragmentation of groups of duckweed fronds also can be a result of disturbance by anthropogenic activity, especially by recreational equipment—trailered boats, rafts, etc. (Rothlisberger et al. 2010). Propagules of duckweeds adhere to their propellers and surfaces and can be transferred into water bodies not only of the same but also to other hydrological systems; this is anthropochory.

Lemna aequinoctialis is registered in the IUCN Red List, version 3.1 (2017) with Conservation Criteria LC (least concern) (http://www.iucnredlist.org/details/164404/0). As an alien invasive species that poses a significant threat to the biodiversity of water ecosystems outside of its natural range, *L. aequinoctialis* is listed in the EPPO Global Database (https://gd.eppo.int/taxon/LEMTN). According to these data (cf. Invasive Species Compendium [CABI, 2021]; https://www.cabi.org/isc/search/index?q=Lemna%20aequinoctialis&page=1&s0=0&s1=10), *L. aequinoctialis* poses also threat to the water ecosystems of countries in tropical and subtropical regions of the planet. The mentioned danger is due to the fact that under favorable environmental conditions, *L. aequinoctialis* has high RGRs even among other *Lemna* species. Therefore, we measured the RGR of all clones of *L. aequinoctialis* and *L. minor* described in the manuscript that originated in Ukraine (Table 4). The RGR of the two clones of *L. aequinoctialis* were 0.409 d^{-1}. Ziegler et al. (2015) investigated three clones of this species from the United States, Venezuela, and India having RGR between 0.473 and 0.478 d^{-1}. This means that the growth rates of the two Ukrainian clones are slightly lower. The six clones of *L. minor* from Ukraine have RGR between 0.351 and 0.398 d^{-1} (Table 4), whereas three clones of this species from the United States, New Zealand, and Germany have RGR between 0.374 and 0.426 d^{-1} (Ziegler et al., 2015). Thus, the presently available data tell us that the *L. minor* clones from Ukraine have

TABLE 3 Results of combined morphological and molecular identification of *Lemna* samples from Ukraine

Clone ID	*Genus*	*Species*
KJA018	*Lemna*	*aequinoctialis*
KJA019	*Lemna*	*aequinoctialis*
KJA022	*Lemna*	*minor*
KJA036	*Lemna*	*minor*
KJA037	*Lemna*	*minor*
KJA038	*Lemna*	*minor*
KJA039	*Lemna*	*minor*
KJA040	*Lemna*	*minor*

Note: For the place of origin, cf. Table 1.

TABLE 4 Growth rates of *Lemna* species collected in Ukraine

Clone ID	Species	RGR (d^{-1})	DT (d)	RY ($g\,g^{-1}$ $week^{-1}$)
KJA018	*Lemna aequinoctialis*	0.409 ± 0.008	1.70 ± 0.03	17.6 ± 1.0
KJA019	*Lemna aequinoctialis*	0.409 ± 0.010	1.70 ± 0.04	17.7 ± 1.3
KJA022	*Lemna minor*	0.398 ± 0.010	1.75 ± 0.05	16.4 ± 1.1
KJA030	*Lemna minor*	0.372 ± 0.008	1.87 ± 0.04	13.6 ± 0.7
KJA037	*Lemna minor*	0.364 ± 0.016	1.93 ± 0.10	13.1 ± 1.3
KJA038	*Lemna minor*	0.393 ± 0.005	1.76 ± 0.02	15.7 ± 0.6
KJA039	*Lemna minor*	0.368 ± 0.016	1.90 ± 0.02	13.6 ± 0.1
KJA040	*Lemna minor*	0.352 ± 0.017	1.20 ± 0.10	12.2 ± 1.5

Note: Relative growth rates (RGR, d^{-1}), doubling time (DT, d) and relative yield (RY; g g^{-1} $week^{-1}$) were measured and calculated according to Ziegler et al. (2015). For further explanations, see Tables 1 and 2.

similar RGR to clones from different places of the world. It should be mentioned that the alien clones of *L. aequinoctialis* in Ukraine have significantly higher RGR than the common clones of *L. minor*. However, the present investigations were carried out only at the standard temperature of 25°C. Survival and competition with other species depend on behavior at different temperatures over the seasons of the year, and this has not yet been investigated by us (Paolacci et al., 2021). Moreover, *L. aequinoctialis* often blooms and bears fruit, in contrast to *L. minor*, which may have consequences for dispersal (Landoldt, 1986).

Lemna aequinoctialis is a species of practical importance, as a food and technological resource, as well as for wastewater treatment from various pollutants and biotesting of water quality (Acosta et al., 2021). This species was used for toxicity tests of such elements as Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb, Zn, Se, and U (Pease et al., 1999) and also of a wide spectrum of herbicides and other xenobiotica (Michel et al., 2004; Ziegler et al., 2016; Mooney & van Dam, 2016). *Lemna aequinoctialis* can be used for phytoremediation of Mn and other cations (Zhou et al., 2019). This species is effective for treatment of wastewater to remove phosphorus and nitrogen (Zhao et al., 2014). It has shown removal efficiencies of 66%, 48%, and 77% for NH_4^+, total N, and total P, respectively.

It was reported (Yu et al., 2014) that *L. aequinoctialis* (strain 6000) had a fast growth rate and the ability to accumulate high levels of starch. After 24 days of cultivation, the starch content increased from initially 28% to 40% on a dry weight basis. Therefore, *L. aequinoctialis* showed great potential as a feedstock for producing starch-based products such as fuel ethanol (Appenroth et al., 2021). After enzymatic scarification and fermentation, starch in *L. aequinoctialis* was easily and efficiently converted into ethanol. High levels of starch production by this species were also reported by Ma et al. (2018). These features enable the use of this species even as a "green fertilizer" (Sengupta et al., 2005).

However high intensity of invasion of *L. aequinoctialis* into natural aquatic biotopes can lead to negative consequences like fish mortality and decrease in invertebrate diversity as shown in Malawi (Lancar & Krake, 2002).

3.1 | Concluding remarks

Given the available literature data, 12 species of the genus *Lemna* L. are known in general (Bog, Appenroth, & Sree, 2020; Bog, Sree, et al., 2020). In the Checklist of vascular plants of Ukraine (Mosyakin & Fedoronchuk, 1999), four species of the genus *Lemna* were reported: *Lemna gibba*, *L. minor*, *L. trisulca*, and *L. minuta*. Moreover, two more invasive species were described in recent years: *Lemna minuta* Kunth (reported under the synonym *L. minuscula* Herter) by Dubina and Protopopova (1983) and *Lemna turionifera* by Orlov and Yakushenko (2013). In our present study, another alien species of duckweed, found in the north of Ukraine (vincinity of Levkiv village, Zhytomyr region), was identified by a combination of morphological and molecular methods. *Lemna aequinoctialis* Welw. represents a new alien species of duckweed for Ukraine. In the future, it could possibly become an invasive species in the country. Thus, at the present time in Ukraine, six species of the genus *Lemna* are known, three native and three alien species.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Tetiana Fedoniuk https://orcid.org/0000-0002-6504-0893
Manuela Bog https://orcid.org/0000-0002-9979-8336
Oleksandr Orlov https://orcid.org/0000-0003-2923-5324
Klaus Juergen Appenroth https://orcid.org/0000-0002-6385-6645

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