The evolution of the duckweed ionome mirrors losses in structural complexity

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ABSTRACT

- **Background and Aims:** The duckweeds (Lemnaceae) consist of 36 species exhibiting impressive phenotypic variation, including the progressive evolutionary loss of a fundamental plant organ, the root. Loss of roots and reduction of vascular tissues in recently derived taxa occur in concert with genome expansions of up to 14-fold. Given the paired loss of roots and reduction in structural complexity in derived taxa, we focus on the evolution of the ionome (whole-plant elemental contents) in the context of these fundamental body plan changes. We expect that progressive vestigiality and eventual loss of roots may have both adaptive and maladaptive consequences which are hitherto unknown.

- **Methods:** We quantify the ionomes of 34 accessions in 21 species across all duckweed genera, spanning 70 million years in this rapid cycling plant (doubling times are as rapid as ~24 hours (Lam & Michael, 2022)). We relate both micro- and macroevolutionary ionome contrasts to body plan remodelling and show nimble microevolutionary shifts in elemental accumulation and exclusion in novel accessions.

- **Key Results:** We observe a robust directional trend in calcium and magnesium levels decreasing from the ancestral representative *Spirodela* genus towards the derived rootless *Wolffia*, with the latter also accumulating cadmium. We also identify abundant within-species variation and hyperaccumulators of specific elements, with this extensive variation at the fine – as opposed to broad – scale.

- **Conclusions:** These data underscore the impact of root loss, and reveal the very fine scale of microevolutionary variation in hyperaccumulation and exclusion of a wide range of elements. Broadly, they may point to trade-offs not well recognized in ionomes.

Key words: vestigiality; duckweed; ionomics; evolution; ICP-MS; *Spirodela*; *Landoltia*; *Lemna*; *Wolffiella*; *Wolffia*

INTRODUCTION

The duckweeds (Lemnaceae) consist of 36 species exhibiting broad variation, including in recently derived species the progressive evolutionary loss of a fundamental plant organ, the root. This progressive loss of roots is accompanied by an overall reduction in vascular tissues in derived taxa. Given the paired loss of roots and reduction in structural complexity, we here focus on the evolution of the ionome and place it in the context of these fundamental body plan changes.

Consisting of five genera progressively differing in root number and vascular complexity, the duckweeds present broad variation in highly simplified body plans (Figure 1). The earliest diverged lineages, *Spirodela* and *Landoltia* (Figure 1, top), were originally both considered Spirodela, but are now recognized as distinct (Les & Crawford, 1999; Les *et al.*, 2001; Bog *et al.*, 2015). The three more recently diverged genera, *Lemna*, *Wolffiella* and *Wolffia*, represent novel forms, with progressively diminished roots and reduced vascular tissues (called nerves) - or none at all (Figure 1, bottom; Appenroth *et al.*, 2013; Tippery *et al.*, 2015). The divergence time between rooted *Spirodela polyrhiza* and rootless *Wolffia australiana* is estimated at 70 million years (Park *et al.*, 2021). Since this divergence, at least 36 duckweed species have formed (Appenroth & Sree, 2020; Bog *et al.*, 2020), which vary 14-fold in genome sizes (Hoang *et al.*, 2019). The smallest is an Arabidopsis-scale 158 Mb genome in *Spirodela polyrhiza* (Wang *et al.*, 2011; An *et al.*, 2018), with the largest genomes in the derived *Wolffia*, exhibiting a radically simplified body plan and diminished vasculature, and no roots (Figure 1 bottom row; Park *et al.*, 2021; Yang *et al.*, 2021).

In contrast to vascular land plants, duckweeds have miniscule bodies in direct contact with water and limited to non-existent root systems. This results in small distances for ion

translocation (Zhang *et al.*, 2009). However, the relative differences in translocation distance can be large: frond sizes of *Spirodela* are >1 cm, but only <1 mm in *Wolffia*. Duckweed roots are considered adventitious, lacking lateral roots and root hairs (An *et al.*, 2019a). Root-forming species have flexibility in their root systems which may develop or elongate in stress situations or drop off (Landolt, 1986). Root functions in anchorage, aggregation to form duckweed mats and aiding dispersal by attachment have all been proposed (Cross, 2017; Ware *et al.*, 2023). Indeed, in the highly derived Wolffioideae the shrinking of body size and complete root loss have evolved to maximize growth rate, improve mobility, and enhance adaptability to changing environments (Wang *et al.*, 2010; Michael *et al.*, 2020; Yang *et al.*, 2021). We expect that duckweeds, representing this unique example of progressive root reduction through to complete loss, would illustrate a gradient of phenotypic changes resulting in altered internal macronutrient and trace element compositions (Fang *et al.*, 2023; Ware *et al.*, 2023).

At the fine scale, duckweed habitats differ in their availability of elements; thus adaptation of accessions to their environments can occur through different elemental storage and exclusion strategies (Mkandawire & Dudel, 2007; Zhang *et al.*, 2009; Van Dam *et al.*, 2010; Lahive *et al.*, 2011). Indeed, duckweed tolerance to elemental extremes is an important trait driving adaptive – and sometimes strongly invasive – strategies in the wild (Wang, 1991; Naumann *et al.*, 2007; Ekperusi *et al.*, 2019). To date, however, the tolerance of only a handful of duckweed accessions to external elemental concentrations has been assessed, with reports focusing on growth vigour vis-à-vis single elements in *Lemna* and *Landoltia* species. Studies quantifying elemental composition are rare, with the broadest study looking at only a single genus, *Wolffia*, with 11 species assessed (Appenroth *et al.*, 2018). We collected existing reports of duckweed elemental variation; however, serious confounding factors

plague interpretation of different studies, due to discordant methods and quantification (Table 1).

Here we bridge this gap, reporting whole-plant ionome compositions in 34 duckweed accessions spanning 21 species and representing the worldwide range of all five duckweed genera (Figure 2; Supplementary Table 1). We place these data into an evolutionary context, focusing on 11 key macro-, micro-, and trace elements, contrasting microevolutionary variation (accession level, within species variation) with macroevolutionary (between genera) trends. These results reveal extensive ionomic variation at both the within-species and between-genus levels, with particularly clear trends for Ca and Mg accumulation differences, as well as possible excess Cd accumulation in the rootless *Wolffia/Wolffiella*. We discern a broad evolutionary trajectory toward very low levels of essential Ca and Mg—as well as increased Cd accumulation—in the recently derived rootless species. This suggests a potentially deleterious consequence associated with the root loss and body-wide vasculature reduction.

MATERIALS AND METHODS

Plant Growth and Care. Duckweed accessions were grown in axenic conditions from single isolates or 5-10 individuals, depending on size of duckweeds, in 100 ml nutrient media (N medium) in individual sealed sterile glass conical flasks. Duckweeds were sourced from the Landolt Collection (now housed in Milan). N medium was described in Appenroth *et al*, 1996 (KH_2PO_4 (0.15 mM), $Ca(NO_3)_2$ (1 mM), KNO_3 (8 mM), $MgSO_4$ (1 mM), H_3BO_3 (5 µM), MnCl2 (13 µM), Na_2MoO_4 (0.4 µM), FeEDTA (25 µM). Element concentrations of supplied N medium including presence of other trace elements were measured by ICP-MS and is presented in Dataset S1. Weekly media changes were performed, with rinses in Milli-Q

(Millipore) water to regulate nutrient composition availability. Plants were grown at 100 µmol m^{-2} s^{-1} under broad spectrum (white) LED lights at 22°C/18°C with a 16 h day/night cycle. Four-week-old duckweed cultures were washed on plastic sieves using a three-step protocol two minutes each of Milli-Q (Millipore) water, CaCl2 and Na-EDTA and harvested into individual samples from flasks of individual populations. These were harvested for ICP-MS analysis on day one, three and five after media change, *n*=6 per time point. Four-week-old cultures are clonally reproduced and therefore suitable replicates, given the very low generational variation and low mutation rates shown in duckweed mutation accumulation experiments (Xu *et al.*, 2019).

Imaging and Microscopy. All samples were cleared and then stained with Fluorescent Brightener 28 (calcofluor) following the protocol described by (Kurihara et al., 2015) and imaged on a Leica TCS SP5 confocal microscope. In short, plants were cleared, based on the ClearSee procedure described by Kurihara et al. (2015), modified slightly. As fluorescent markers were not being used, plants were fixed overnight in ethanol and acetic acid (3:1 v/v) rather than paraformaldehyde, as this reduced the toxicity and requirement for vacuum infiltration, which can be damaging to the air spaces. Plants were then rinsed three times in RO water and left for 30 min, then RO water was replaced with ClearSee solution (10% Xylitol, 15% Sodium Deoxycholate, 25% Urea; Kurihara et al., 2015) and left to clear for 2 weeks. Prior to imaging, plants were stained for 1 h with calcofluor in ClearSee (100 µg/ml), and then washed in ClearSee for 1 h. Imaging was carried using a confocal laser scanning microscope (Leica SP5), using a 405 nm diode laser at 12% and hybrid detector with a range of 440–450 nm, gain of 25%, and pinhole of 0.5 AU.

Quantification of elemental tissue concentrations. For ICP-MS we used a method adapted from Danku et al., 2013. Briefly, 5-20 mg (fresh weight) was harvested per sample, placed in Pyrex test tubes and dried at 88°C for 24 h. Then the dry weight was recorded and 1 ml concentrated trace metal grade nitric acid Primar Plus (Fisher Chemicals) spiked with an internal standard was added to the samples that were further digested in DigiPREP MS dry block heaters (SCP Science; QMX Laboratories) for 4 h at 115°C. Prior to the digestion, 20 µg/ L of Indium (In) was added to the nitric acid as an internal standard for assessing errors in dilution, variations in sample introduction and plasma stability in the ICP-MS instrument. Then 0.5 ml of hydrogen peroxide (Primar, for trace metal analysis, Fisher Chemicals) was added to the samples and they were digested for additional 1.5 h at 115°C. After digestion, samples and blanks were diluted to 10 ml with Milli-Q (Millipore). Direct water and elemental analysis was performed using an ICP-MS, PerkinElmer NexION 2000 with 22 elements monitored (Li, B, Na, Mg, P, S, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd and Pb) in the collision mode (He). To correct for variation between and within ICP-MS analysis runs, liquid reference material was prepared using pooled digested samples, and run after every nine samples in all ICP-MS sample sets. The calibration standards were prepared from single element standards solutions (Inorganic Ventures; Essex Scientific Laboratory Supplies Ltd, Essex, UK). Sample concentrations were calculated using external calibration method within the instrument software. Further data processing including calculation of final elements concentrations (in mg/kg) was performed in Microsoft Excel. Log transformations, Z-score calculations and graphical representation was performed using R (version 3.0.2 "Frisbee Sailing"; R Development Core Team, 2013; see http://www.R-project.org) and RStudio v 1.0.136 (RStudio Team, 2020) was used for all statistical analyses. To calculate relationships between elements the corrplot package (McKenna *et al.*, 2016) was used in R with Pearson correlations on log10 transformed data.

RESULTS

Broad scale evolution of the ionome

We focus on ionomes from day five after media change (Figure 3), which is representative of other time points (None of the 11 elements upon which we focus were significantly different across days by ANOVA). The full raw data set is given in Dataset S2; elements we considered for further analysis shown in Supplementary Figure 1. Concentrations were consistent for all elements for all accessions between time points except for a handful of elements in certain accessions depicted in Supplementary Figure 2. These exceptions show a small minority of accessions decreasing in K, Ca, Fe and Cd and others still increasing e.g. Ca, Cu, Fe. For accumulators showing the latter pattern, for example *Sp. intermedia* 9227, maximum concentration capacity of Ca on day one after media changes was still not reached, despite high nutrient provision throughout a four-week experimental period, and the accession could still prolong uptake.

In the overall dataset of 34 accessions, the broadest contrast observed was between the Lemnoideae and Wolffioideae (rooted and rootless, respectively) for Ca, Mg and Cd accumulation (Figure 3a). All ancestral representatives (rooted) Lemnoideae (*Spirodela*, *Landoltia* and *Lemna*) consistently exhibit 2-3x higher Ca content relative to the derived rootless Wolffioideae ($p =<0.01$; log10, ANOVA with post-hoc Tukey test). Similarly, on average, Mg accumulation was 1.8x higher in the rooted species relative to the rootless *Wolffia* and *Wolffiella*. Ca and Mg show a positive correlation (Table 2, Supplementary Figures 3 and 4). We observed further variation for Mg in the *Lemna* genus, where there emerged a gradient of Mg accumulation across *Lemna* sections (Figure 1, Figure 3 a, d). The highest Mg levels were in the Uninerves section (Figure 3a, Figure 4), which includes the invasive *Lemna minuta* and *Lemna yungensis* (now *L. valdiviana*), as described in

(Tippery *et al.*, 2015 and Bog *et al.*, 2020) and both alien within Europe (Kirjakov & Velichkova, 2016; Ceschin *et al.*, 2018). This association of Mg accumulation with increased root vasculature (as well as reduced frond vasculature in *Lemna*) stands in strong contrast to the uniformly very low Mg in rootless Wolffioideae. Cadmium concentrations varied significantly between rooted and non-rooted duckweeds (Figure 3a; $p < 0.05$; log 10, ANOVA with post-hoc Tukey test) in a manner inverse to Ca and Mg. The unrooted Wolffioideae species (especially *Wolffiella*) showed the highest Cd concentrations. Only the submerged *Lemna trisulca* exhibited comparably high Cd to the Wolffioideae (Figure 3).

Rootless species exhibiting variation in at least two elements included *Wolffiella lingulata*, *Wolffiella hyalina*, and *Wolffia brasiliensis* (Figure 3e). In contrast, the species in our panel from the multi-rooted more ancestral duckweed representatives *Spirodela* and *Landoltia* showed the greatest ionomic consistency across all accessions (Figure 3b). *Spirodela* species had the highest Ca tissue content in our panel, but other elements were not as variable between accessions.

Fine scale ionome variation and identification of extreme accumulators in Lemna

We observe the greatest within-genus ionome variation in the *Lemna* genus (n=20 accessions, 6 biological replicates of each; Figure 3c-d). *Lemna* also harbours several extreme accumulators, each standing as outliers for the accumulation of three or more elements. *Lemna trisulca* 7192 has a submerged growth pattern and accumulated the greatest number of elements in amount and number from the panel, showing very high tissue concentrations of four essential elements: P, Ca, Zn, Fe as well as Cd, and low K levels (Figure 3d). *Lemna yungensis* 9210 accumulated high S and Mn, and also exhibited low K (Figure 3c). Indeed, K

levels trend negatively against the enhanced accumulation of other macro- and microelements in both *Le. trisulca* and *Le. yungensis* and across our panel as a whole (Table 2; Supplementary Figure 4).

Fine scale ionome variation between Lemna species

At the within-species level we note variation at the level of several accession pairs, most obviously between *Le. yungensis* accessions (Figure 3c). Notably, *Le. yungensis* 9208 greatly accumulated Mg, and *Le. yungensis* 9210 exhibited extreme accumulation of S and Mn, but low K. When comparing *Le. yungensis* with *Lemna valdiviana* clones, none of the accessions showed large differences in ionomes between ten elements, with consistent levels of B and S (Figure 5a). Comparing *Lemna minor* with *Lemna turionifera* and their interspecific hybrid *Lemna japonica*, *Le. japonica* accessions had lower Mo and a slight increase in Na and K in specific *Le. japonica* clones (Figure 5b); however, neither of these ionome changes were significant compared to the whole duckweed panel. When contrasting native European *Le. minor* clones with invasive European *Le. minuta*, we see clone-level variation in some elements, but none were significantly varied from the overall population by as much as one SD (Figure 5c).

DISCUSSION

The broad variation we observed in duckweed ionomes at levels of genera, species, and sister accessions is presumably in large part due to both morphological differences and adaptation to micro-environments. The most robust differences were at the genus level for Ca, Mg and Cd. The accumulation difference for Ca is perhaps explained in part by a storage mechanism

as Ca oxalate (CaOx) within frond crystal ultrastructures in rooted genera, in the fronds of *Spirodela* and *Lemna*, (Landolt & Kandeler, 1987) and also in the root of *L. minor* (Franceschi, 1989, Mazen *et al.*, 2003). In *Le. Turionifera*, Ca influxes through roots and is stored in both fronds and roots, and in exceptional cases can also be effluxed out of roots (Ren *et al.*, 2022). In contrast, Wolffioideae species have soluble Ca in cell sap and accordingly also cannot store excess Ca in roots (Landolt & Kandeler, 1987; Appenroth *et al.*, 2017); thus Ca and Mg may be lower in Wolffiodeae as they lack roots as a storage organ. As Ca was kept sufficiently available in our experiment through media refreshes, and rooted duckweeds use their roots as an additional storage compartment (Ren *et al.*, 2022), this may result in overall higher accumulation when compared with their rootless counterparts.

Given the broad contrasts in Ca between genera, it is interesting to consider these results alongside the importance of roots for elemental uptake and segregation of individual elements between frond and roots in duckweed species. The excision of roots makes only a modest change to the frond ionome, showing roots are vestigial and overall not required for nutrient uptake in replete media conditions (Ware *et al.*, 2023). This supports the notion that duckweed roots may be adventitious (Landolt, 1986; An *et al.*, 2019b). Whilst removal of roots surprisingly increased elemental composition in some cases (Ware *et al.*, 2023), the picture is more complicated as rootless species do not naturally exhibit elevated Mg or Ca in our data, indicating evolutionary adjustment of ion homeostasis upon root loss. Indeed, the *Wolffia* genome harbours a derived complement of Ca export and cell wall thickening genes, possibly minimizing potential for apoplastic transport, which coupled with inability for storage as CaOx, results in less specialised mechanisms to manoeuvre and store Ca content overall (Michael *et al.*, 2020). In contrast, clones of *Le. aequinoctialis*, *Le. minuta* and *Le. minor* exhibit marked Ca accumulation (storage) to alleviate Mg toxicity from a contaminated

mine and on high Mg:Ca ratio media or wastewater (Van Dam *et al.*, 2010; Paolacci *et al.*, 2016; Walsh *et al.*, 2020). This suggests specific adaptation of Ca storage and transport mechanisms to particular ionomic challenges.

The Mg gradient across *Lemna* species does not necessarily correlate with strict overall inferred ancestral and derived forms (Wang *et al.*, 2011; Tippery *et al.*, 2015) and root vascular complexity is not sufficiently varied between rooted duckweeds to account for this (Ware *et al.*, 2023). Instead, higher specific Mg uptake in the Uninerves section of *Lemna* may be associated with their reduced frond vascular complexity (Figure 3a, Figure 4). With typical frond nerves up to 16 in number in *Spirodela* and between 3-7 in other *Lemna* species (Les *et al.*, 2002), only one nerve is present in *Le. yungensis* and *Le. minuta*, with *Le. yungensis* (now *Le. valdiviana*) having the longer nerve of the two (Landolt, 1980; Crawford *et al.*, 1996). It is thought that this simplified vascular system may contribute to their invasive status (Kirjakov & Velichkova, 2016; Kadono & Iida, 2022). Reduced vascular complexity and ionomic differences could also offer enhanced potential for adaptation to varied environments, showing higher Mg tolerance (Paolacci *et al.*, 2016) and possibly therefore, survival in hard water.

While some variation in mineral content among *Wolffia* species has been reported by Appenroth *et al*, (2018), *Wolffiella* have received little attention and can be under-reported due to clones having restricted biogeography and not being readily available (Landolt, 1986; Kimball *et al.*, 2003). Therefore, multi-elemental compositions of rooted and rootless duckweeds have not been directly compared as here. In this respect we see relative accumulation of Cd, especially in *Wolffiella* compared to the rooted species. This is

somewhat surprising, as it may be expected that Cd accumulation would be detrimental to minuscule plants with no root segregation away from photosynthetically active tissue. We note, however, that *Wolffia* species also exhibit tolerance to As and have been considered as candidates for phytoremediation, accumulating more than Lemnoideae (Zhang *et al.*, 2009). Additionally, there is good evidence that *Wolffia* has moderate tolerance to Cd and increased accumulation capacity even in extreme concentrations (>200 µM). In fact, a handful of *Wolffia* species show Cd uptake in as little as 30 minutes from solution via apoplast which increases linearly with Cd concentration (Boonyapookana *et al.*, 2002; Xie *et al.*, 2013). We therefore speculate that loss of roots could have reduced control of heavy metal uptake whilst at the same time roots loss removes a potential mechanism of uptake and a storage compartment available to rooted species (Verma & Suthar, 2015; Ma *et al.*, 2023; Zheng *et al.*, 2023).Wolffioideae perhaps evolved higher tolerance mechanisms to Cd toxicity e.g. compartmentalisation to vacuoles and complexation via conjugates (Schreinemakers, 1986). Although Cd was not supplied in a dedicated quantity in N medium preparation, we quantified Cd presence by ICP-MS in the media used (Dataset S1) and suggest this comes from chemical impurities as indicated in (Appenroth *et al.*, 2018). We infer that *Wolffioideae* species may have a potential for heavy metal accumulation at greater dosages than here, perhaps also in the wild through adaptation to contaminated habitats (Zhang *et al.*, 2009).

Our results show that the genus with the greatest diversity of specific accumulators was *Lemna*. The *Lemna* accessions with most extreme ionomes, *Le. trisulca* 7192 and *Le. yungensis* 9208, also harbour the most divergent root architecture, compared to other *Lemnas*. *Lemna trisulca* is characterised by a submerged growth habit but smaller cortical cells giving a thin, reduced root compared to other *Lemna* species, and *Le. yungensis* 9208 often displays an additional layer of cortical cells and irregularly large extracellular airspaces in the root

cortex (Ware *et al.*, 2023). Thus, these differential root vasculature components, coupled with minimal frond vasculature, may play a role in producing the contrasting elemental profiles observed. Both *Le. trisulca* and *Le. yungensis* accumulated over 1000 mg/kg dry weight for several elements and can therefore be considered hyperaccumulators (Zayed *et al.*, 1998; Zhang *et al.*, 2009). For this reason, these two species may have potential to be used in combination to alleviate multi-elemental toxicity in watercourses. *Lemna trisulca* accumulated greater Zn and Cd than floating species, possibly because of increased absorption through submerged fronds. Although *Le. trisulca* had the greatest variation overall and maximal micronutrient levels, the associated high Cd accumulation may be problematic for any applications in nutrition. It is also unclear whether this trait is common in other *Le. trisulca* accessions due to limited availability of clones in stock centres, however this species has previously been noted for its Cd accumulation potential (Kara & Kara, 2005).

A greater appreciation for duckweed variation in micronutrients Ca, Mg, Fe and Zn is clear from our study, with particular accessions acting as hyperaccumulators for multiple nutritionally-relevant elements. This is not the case for trace elements such as Na and Cu (and especially Mn and the heavy metal Cd), where tissue concentration variation was less dramatic than seen in other reports (Table 1). This is likely due to the combined effect of low presence of these elements in our supplied media or that comparisons across literature are confounded by variables disallowing truly quantitative comparisons between studies. This is particularly evident for Cd, which we supply only in trace amounts (Dataset S1), whereas external Cd concentrations vary 500-fold between studies.

Synthetic biology, including the tailoring of ionomic profiles in duckweeds, is an important goal of the duckweed research community (Lam & Michael, 2022). Interestingly, the *Spirodela* genome sizes are the smallest and the ionomes the least variable among all

duckweeds here (Wang et al., 2011; An et al., 2018); additionally, the amenability of *Spirodela* to genetic transformation (Yang *et al.*, 2018a; Yang *et al.*, 2018b) makes it a strong candidate as a minimal scaffold for synthetic biology. We additionally suggest that because their ionomic profiles are so variable, the larger genome-harbouring species will be particularly valuable to mine natural variation to inform transgenic approaches in the smaller, highly tractable *Spirodela* genome.

For the fine scale variation between *Lemna* species of interest, the vast ionome differences between *Le. yungensis* 9208 and 9210 can be best ascribed to local adaptation. Given these accessions are closely related and were both originally isolated from the same region in Bolivia, one might expect more similar ionome profiles, but instead our data show that duckweeds exhibit strongly contrasting local variation in elemental uptake. Interestingly, this region of Bolivia is reported as atypically harsh for duckweed, growing on sheer rock faces with waterfall spray with low nutrient availability (Landolt, 1998). It will be valuable to characterise *Le. yungensis* species further, in order to determine the genetic basis for their adaptation to specialised habitats. As *Lemna yungensis* and *Lemna valdiviana* showed no other significant internal differences between ten elements, this supports their unification as one species due to lack of genetic differentiation (Bog *et al.*, 2020). *Lemna minuta* is an invasive species in introduced regions with ecological significance (Ceschin *et al.*, 2018), as an opportunist species in replete N and P with additional higher Mg tolerance (Njambuya *et al.*, 2011; Paolacci *et al.*, 2016; Ceschin *et al.*, 2020) one would expect drastic differences in the ionome in comparison to *Le. minor*. Despite this, there were no clear pattern differentiating two *Le. minuta* from two *Le. minor* clones grown nutrient rich medium (N medium; Appenroth *et al*, 1996; measured here in Dataset S1). Elemental differences seem to be at the clonal level and opportunism therefore probably depends on unique situations in the wild. Recent data classified *Le. japonica* as a hybrid between *Le. minor* and *Le. turionifera*

(Braglia *et al.*, 2021; Volkova *et al.*, 2023). Hybrid *Lemna japonica* clones had slightly reduced Mo compared to parents and one clone had significantly higher Na. It could be that hybridisation may result in ionome differences important for altered adaptation to varied environments, as found in other plant species (Arnold *et al.*, 2016; Wong *et al.*, 2022). Taken together, between these groups of *Lemna* species, subtle interspecies differences for elements were clear. The physiological differences between species and their clones in light of genetic differences deserve future attention in duckweed.

CONCLUSIONS

Here we detailed broad- and fine-scale diversity for the accumulation of physiologically and nutritionally important elements across all five duckweed genera. This variation is associated with dramatic morphological reductions in fundamental plant organs and genome expansions. Thus, disentangling the concurrent effects of dramatic genome size expansions, organ reduction, and ecological adaptations will be a great challenge. However, at the more microevolutionary scale, within-species, accession-level variation points to clear promise in mapping alleles responsible for this observed variation.

One might speculate that the observed ionomic changes may be a maladaptive spandrel associated with root loss in derived taxa, but it is hard at this point to identify what the exact trade-off may be; this is for dedicated mechanistic and ecological work on the rootless taxa. Beyond highlighting these enigmatic correlates of root loss and the consequences of organ loss and vestigiality, this work serves to establish phenotypic variation across the ionome at both the fine and broad scale. This serves as a basis for future genomic characterisation of causal alleles, as well as rational development of targeted duckweed lines for both important nutritional and phytoremediation goals.

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DATA ARCHIVING

The data are given as supplemental information to the article.

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FIGURE LEGNEDS

Figure 1. Trajectory from ancestral root-harbouring duckweeds, via vestigiality, to root loss. Ancestral form (above) represented by Lemnoideae: *Spirodela, Landoltia, Lemna*. Derived from (below) shown in Wolffioideae subgroup genera *Wolffiella* and *Wolffia*. All samples were cleared, and stained with Fluorescent Brightener 28 (calcofluor) following the protocol described by (Kurihara *et al.*, 2015) and imaged on a Leica TCS SP5 confocal microscope. Scale bars: *Spirodela, Landoltia* = 1000 µm; *Lemna, Wolffiella* = 500 µm; *Wolffia* = 100 µm. Cladogram schematic topology based on Tippery *et al*., 2015.

Figure 2. Sampling of worldwide duckweeds for ionomic panel. Dots indicate sample origin locations: *Lemna*=green, *Landoltia*=yellow, *Spirodela*=black, *Wolffiella*=orange, *Wolffia*=blue. Duckweeds were derived from the Landolt collection, now housed in Milan.

Figure 3. The evolution of the duckweed ionome across genera, species, and accessions. (a) Relative levels of elemental accumulation across rootless and rooted subgroups, respectively. The heat map is coloured by z-scores for the four most differentially accumulated elements by significant ANOVA Tukey ** significant $p<0.01$ and * $p<0.05$ ANOVA with post-hoc Tukey test differences between Wolffioideae and Lemnoideae. Z-scores (number of standard deviations away from the mean) were generated for each element using log10 transformation of mg/kg values on day 5. X axis s arranged with basal forms on the left and derived on the right. Separating lines indicate genus and subgroup boundaries. We. = *Wolffiella* (2), Wo. = *Wolffia* (5) Le. = *Lemna* (20), La. = *Landoltia* (2) and Sp. = *Spirodela* (5). Within *Lemna* sections Biformes, Alatae, Uninerves and Lemna marked left to right; **(b-e)** Radar plots showing differences in ionome profiles between individual accessions. **(b)** *Spirodela* and *Landoltia*; **(c)** *Lemna* Biformes,

Alatae and Uninerves; **(d)** *Lemna* sect. Lemna; **(e)** *Wolffiella* and *Wolffia* species. Species are ordered in the panels according to (Tippery *et al.*, 2015) with most ancestral representative at top left through to most derived in bottom right. Numbers after species represent clone numbers. Asterisks represent significant increase or decrease +/- 2 relative to all normalised element concentrations for all species based on mean and SD. The complete data set of 17 elements and three timepoints is given in Dataset S2.

Figure 4. Increased Mg content mirrors the reduction of frond vasculature within *Lemna*. Four sections of *Lemna* representing highest Mg content in the species with most reduced vasculature at sect. Uninerves, with transition sects. Biformes and Alatae and most developed frond vasculature in sect. Lemna, with reduced Mg. Mg content is plotted from day five averaged values for each accession within each sect: Uninerves *n*=6, Biformes *n*=2, Alatae *n*=2, Lemna *n*=10. Sections are ordered and described according to (Landolt, 1986; Tippery *et al.*, 2015). Violin plots represent spread of data for each group with middle line plotting mean.

Figure 5. Elements high in N medium show limited differences in internal ionomes between pairs of *Lemna* species. (a) *Lemna yungensis* (now merged with *L. valdiviana*) and *Lemna valdiviana* accessions. **(b)** *Lemna minor*, *Lemna turionifera* and their interspecific hybrid species *Lemna japonica*. **(c)** Accessions of cosmopolitan *Lemna minor* and invasive European alien *Lemna minuta*. Heat maps for Z-scores from day five are presented for each accession. Ten elements were selected based on those intentionally added and present in highest concentrations in N medium. Z-scores +/- 2 SD represent a significant increase or decrease relative to all normalised elements.

TABLES

Table 1. Elemental tissue concentration of duckweeds gathered from the literature. Elements are ordered by type (macro, micro, trace element and heavy metals) reported from the literature and included in our experiment. 1. Appenroth *et al.*, 2018. 2. Mkandawire & Dudel, 2007. 3. Van Steveninck *et al.*, 1992. 4. Khellaf & Zerdaoui, 2009. 5. Lahive *et al.*, 2011. 6. Liu *et al.*, 2017. 7. Prasad *et al.*, 2001. 8. Leblebici *et al.*, 2010. 9. Landolt & Kandeler, 1987.

Element	Species	Fold variation (literature)	Fold variation (this study, 21 species)
P	*Wolffia* spp.	1.7 [1,2]	2.4
K	*Lemna* spp., *Wolffia* spp.	2.4 [1,2]	3.3
Ca	*Lemna* spp., *Wolffia* spp.	3.3 [1,2]	11.4
Mg	*Lemna* spp., *Wolffia* spp.	3.1 [1,2]	19.5
Na	*Lemna* spp., *Wolffia* spp.	29.5 [1,2]	27.4
Fe	*Lemna* spp., *Wolffia* spp.	21.8 [1,2]	111.0
Zn	*Lemna gibba, Lemna minor, Landoltia punctata, Wolffia* spp.	87.3 [1,3,4,5]	149.6
Mn	*Spirodela polyrhiza, Wolffia* spp.	27.3 [1,6]	4.5
Cu	*Lemna trisulca, Lemna gibba, Lemna minor, Wolffia* spp.	15.7 [1,7,8,9]	7.6
Cd	*Landoltia punctata 6001, Lemna minor, Lemna gibba, Spirodela polyrhiza* sp., *Wolffia globosa.*	5,900 [1,10,11,12,13,14]	27.3

Table 2. Mg and Ca correlated strongly and positively whilst K correlated negatively with various elements. Element pairs significantly correlated across 34 duckweeds at three time points. R values correspond to positive or negative Pearson correlations derived from log10 transformed data for eight elements. Data is given to 2 dp.

Element	R
Fe/K	-0.76
Zn/K	-0.72
P/K	-0.67
Mn/K	-0.59
Mg/Ca	0.59
Fe/Mn	0.58
Zn/Mn	0.58

Figure 1

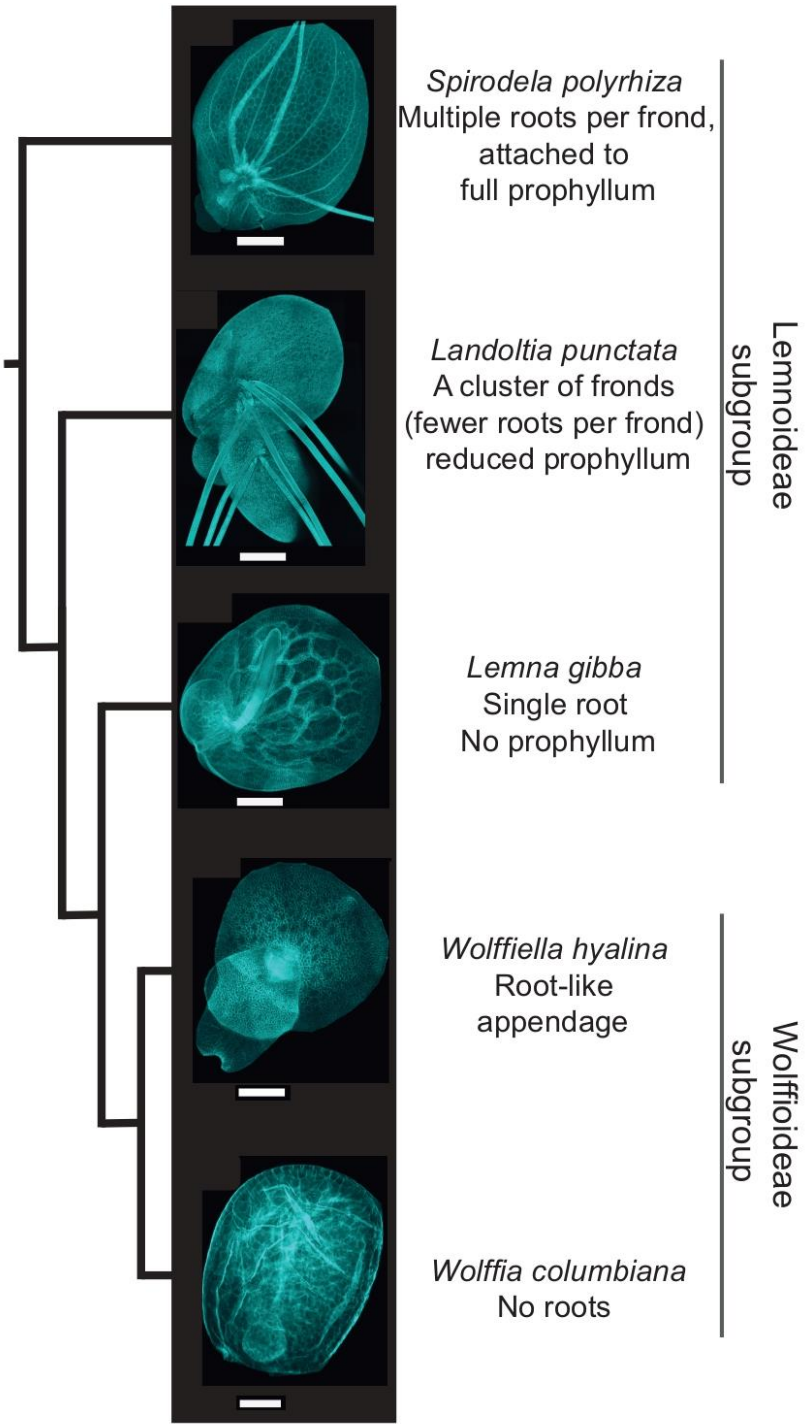

Figure 2

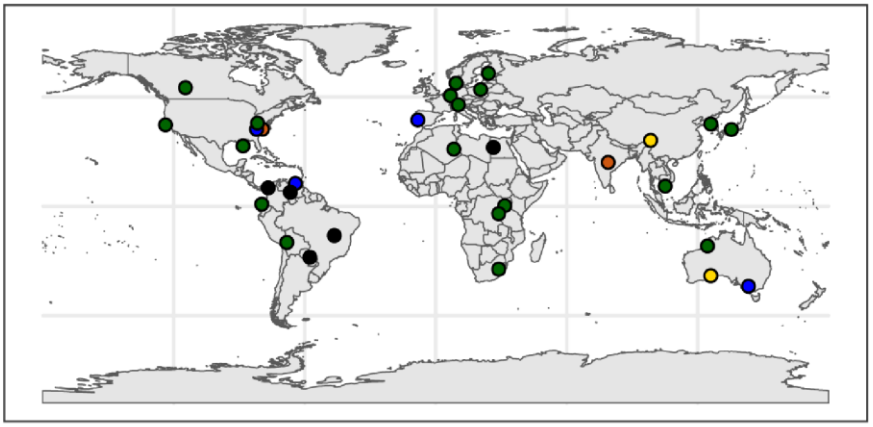

Figure 3

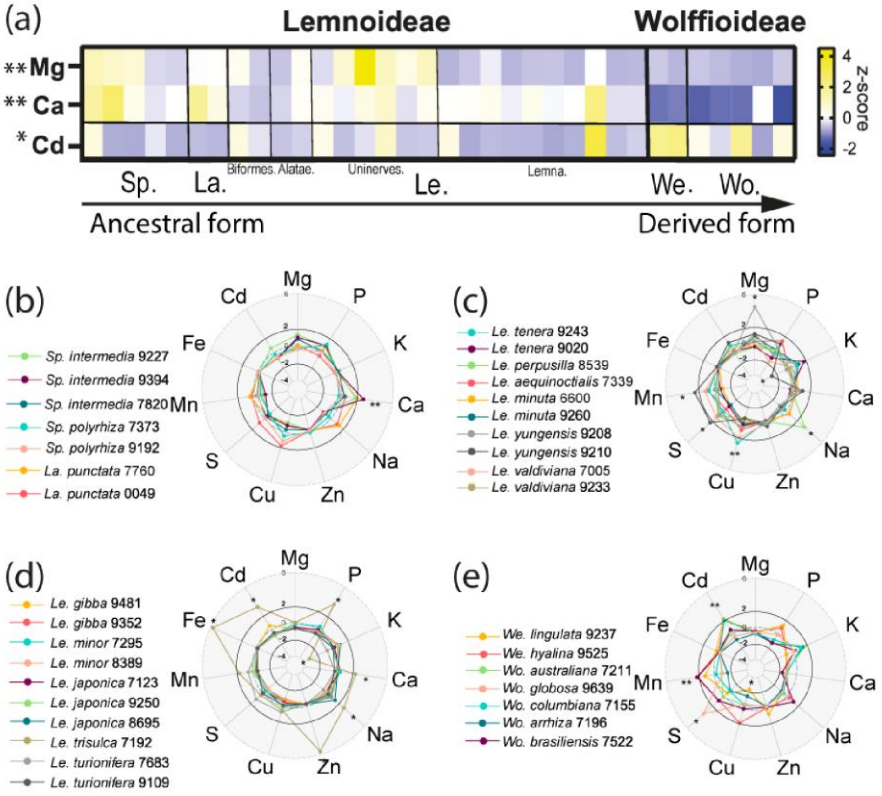

Figure 4

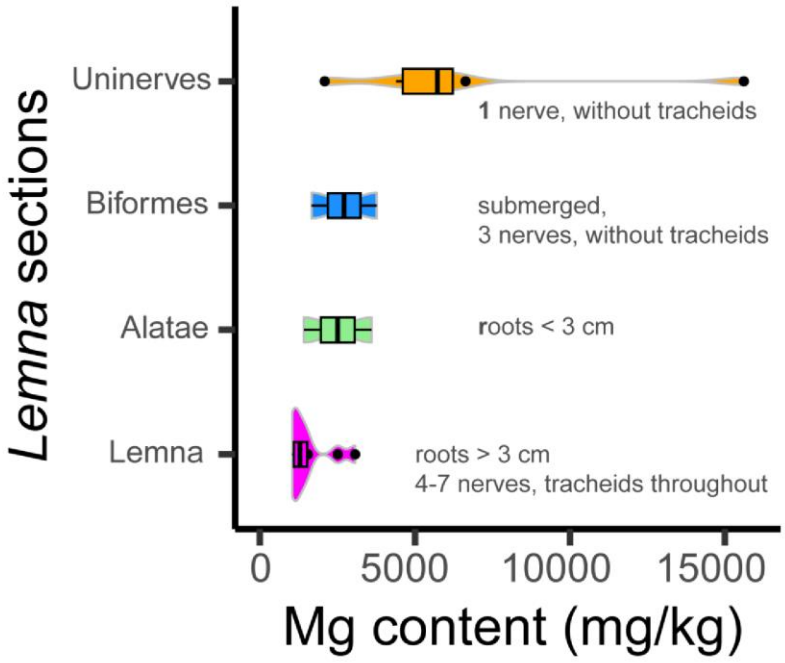

Figure 5