

1 **Title: Duckweed roots are dispensable and are on a trajectory toward vestigiality**

2

3 **Short title: Structural and functional reduction of duckweed roots**

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16 Author contributions

17 AW, BMK & AB designed the concept. AW and DJ performed anatomical analyses and monitored
18 root function with support from JA. PF performed the ionomic profiling. KES aided with sampling for
19 ionomics. AW & AB wrote the manuscript, with input from all authors.

20

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25

26 One sentence summary: Through their adaption to the aquatic environment, duckweed roots have
27 progressively become structurally reduced making them an ideal plant model with which to study
28 vestigiality.

29

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36

37 **Abstract**

38

39 **Duckweeds are morphologically simplified, free floating aquatic monocots comprising both rooted**
40 **and rootless genera. This has led to the idea that roots in these species may be vestigial, but**
41 **empirical evidence supporting this is lacking. Here we show that duckweed roots are no longer**
42 **required for their ancestral role of nutrient uptake. Comparative analyses of nearly all rooted**
43 **duckweed species revealed a highly reduced anatomy, with greater simplification in the more**
44 **recently diverged genus *Lemna*. A series of root excision experiments demonstrated that roots are**
45 **dispensable for normal growth in *Spirodela polyrhiza* and *Lemna minor*. Furthermore, ionic**
46 **analyses of fronds in these two species showed little difference in the elemental composition of**
47 **plants in rooted versus root-excised samples. In comparison, another free-floating member of the**
48 **Araceae, *Pistia stratiotes*, which colonized the aquatic environment independently of duckweeds,**
49 **has retained a more complex root anatomy. Whilst *Pistia* roots were not absolutely required for**
50 **growth, their removal inhibited plant growth and resulted in a broad change in the mineral profile**
51 **of aerial tissues. Collectively, these observations suggest that duckweeds and *Pistia* may be**
52 **different stages along a trajectory towards root vestigialization. Given this, along with the striking**
53 **diversity of root phenotypes, culminating in total loss in the most derived species, we propose**
54 **that duckweed roots are a powerful system with which to understand organ loss and vestigiality.**

55

56 **Introduction**

57 Evolution has shaped the body plans of all organisms into the myriad of diverse forms we see today.
58 While evolution is commonly envisioned as constantly generating novel forms, things sometimes go
59 the other way: occasionally, entire structures or traits are lost, becoming vestigial. This can result in
60 radical shifts in body plan and life-history strategy and is a key evolutionary process driving
61 structural innovation. Based on earlier definitions (Prout, 1964, Fong et al., 1995, Müller, 2002),
62 vestigiality can be broadly defined as the retention, through evolution, of genetically determined
63 structures that have lost some or all of their ancestral function.

64

65 Vestigiality is phylogenetically widespread in plants (Knobloch, 1951). Examples include loss of entire
66 organs, such as floral organs in *Penstemon sp.*, oil glands in *Ceratandra* flowers, leaf reduction in
67 *Equisetum*, and non-functional roots in dodder seedlings, and are often concurrent with atypical,
68 innovative body plans or unusual life history strategies (Walker-Larsen and Harder, 2001; Sherman
69 et al., 2008; Steiner, 1998). To date, reports exploring vestigiality in plants are largely descriptive.

70 Progress into understanding the molecular and evolutionary processes which drive organ loss in
71 plants has therefore been limited.

72

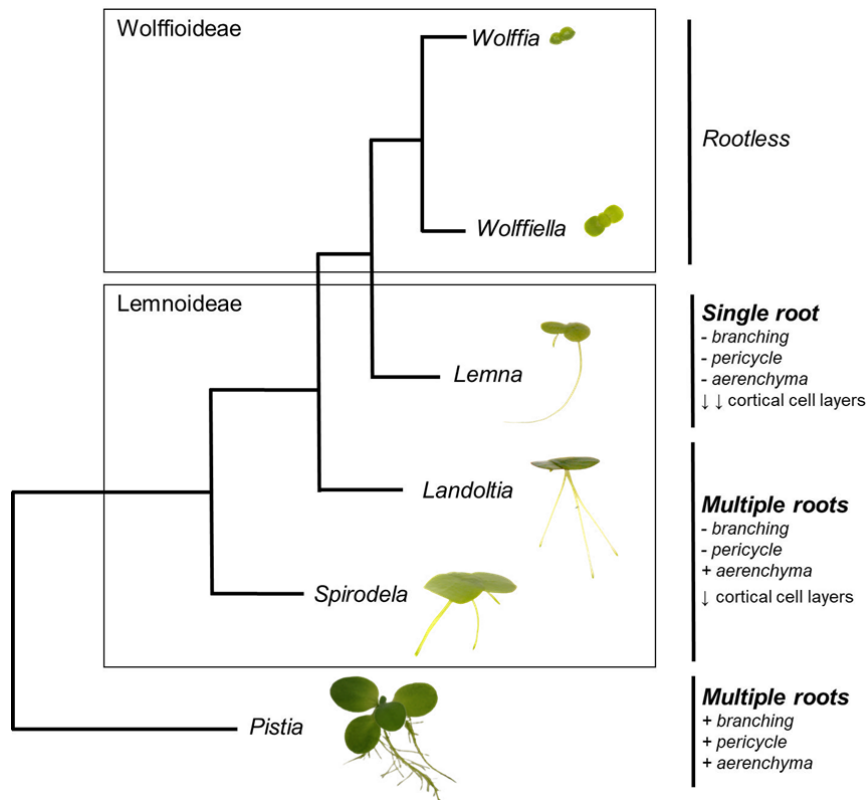
73 The most advanced work driving our understanding of the molecular control of vestigiality comes
74 from outside the plant kingdom. Perhaps the most detailed work has been done on the blind
75 cavefish, *Astyanax mexicanus*, where the mechanisms underpinning eye-loss have been well
76 described. Comparisons between blind and sighted cavefish has revealed that lens apoptosis is
77 mediated by expansion of the expression domain of *sonic hedgehog A* and *B* (*shhA* and *sshB*), which
78 negatively regulate the homeobox gene *pax6*, itself a key regulator of eye development in
79 vertebrates (Yamamoto et al., 2004). It is clear from work in *Astyanax* that leveraging the presence
80 and absence of organs in closely related species is crucial to gaining an understanding of vestigiality
81 at a molecular level. We propose that root loss in duckweeds represents a powerful untapped model
82 for understanding organ loss in plants due to the existence of closely related rooted and rootless
83 species. Recent development of genetic tools (Yang et al., 2018; Vu et al., 2020; reviewed in Acosta
84 et al., 2021) has enabled exploration of molecular networks in duckweeds. However, any study of
85 vestigiality first requires a detailed understanding of how the organ in question functions. As roots
86 are still retained in many duckweed species, we need clarity on duckweed root function to frame the
87 evolutionary context of this model. Within the literature there are several observations regarding
88 the function of duckweed roots; however there is no single study bringing together multiple lines of
89 empirical evidence supporting their vestigiality.

90

91 Duckweeds are highly morphologically reduced free-floating angiosperms lacking many of the key
92 organs common in flowering plants, such as clearly defined stems and leaves. The plant body is
93 reduced to a flattened frond or thallus. They comprise five genera divided into two subgroups,
94 Lemnoideae (*Spirodela*, *Landoltia* and *Lemna*) and Wolffioideae (*Wolffia* and *Wolffiella*). Within these
95 genera, there is an evolutionary trajectory in root number consistent with root vestigialization: the
96 earliest-diverging duckweed genera (*Spirodela* and *Landoltia*) possess multiple roots, later diverging
97 ones a single root (*Lemna*), and the most recently diverging lineages possess no roots at all (*Wolffia*
98 and *Wolffiella*) (Tippery and Les, 2020, Figure 1).

99

100 Duckweed roots are adventitious and neither branch nor form root hairs (Landolt, 1998). Previous
101 studies have performed detailed investigations into root anatomy in individual species, reporting
102 high levels of structural reduction. *Spirodela polyrhiza* roots have a stele comprising of one xylem
103 cell, two sieve elements and between five and six phloem parenchyma cells (Kim, 2007). These are



104 enclosed by a single layer of endodermis, three distinct cortical cell layers and between 38-45
 105 epidermal cells (Kim, 2007). A similar pattern is reported for *Lemna minor* (Echlin, 1981). Although
 106 there have been other studies of root anatomy (eg. Hegelmaier, 1868), we currently miss a
 107 systematic understanding of root anatomy across the three root-bearing genera.

108

109 Vestigialization not only affects anatomy, but also function. It does not imply that organs should
 110 possess no function, only that the salient function is lost. Here, we define the salient function of
 111 roots as organs with which to acquire water and nutrients. Various lines of evidence have been
 112 presented to support the view that duckweed roots have at most a limited role in nutrient uptake.
 113 Hegelmaier (1868) noted that in their natural habitat, individuals of *Lemna gibba* without roots
 114 occur. Gorham (1941) concluded that nutrients were taken up via fronds and not roots, as coating
 115 the underside of fronds with a hydrophobic wax reduced the division rate of fronds and caused root
 116 elongation, whilst coating the upper surface did neither. Muhonen and colleagues (1983) also noted
 117 that *Spirodela polyrhiza* grew without roots. Whilst these studies suggest that roots may not be
 118 required for growth, they do not rule out that duckweed roots still play some role in resource
 119 capture. Indeed, it has been observed that both roots and fronds can assimilate nitrogen in both
 120 *Lemna minor* and *Landoltia punctata* (Cedergreen and Madsen, 2002; Fang et al., 2007).

121

122 The above presents an incomplete picture of vestigiality in duckweed roots. To address this, we
123 conducted a survey of duckweed root anatomy across almost all the rooted duckweed species. We
124 examine to what extent changes in anatomy are consistent with roots being vestigial, and if
125 additional structural reduction accompanies the reduction in root number between genera. We then
126 investigated root function in two species by looking at growth and uptake of 13 elements in plants
127 with and without roots excised. By comparing duckweeds with the related free-floating macrophyte
128 *Pistia stratiotes* we present a scenario in which both anatomical complexity and the role of the root
129 in foraging for nutrients has been progressively lost in duckweeds.

130

131

132 **Results**

133

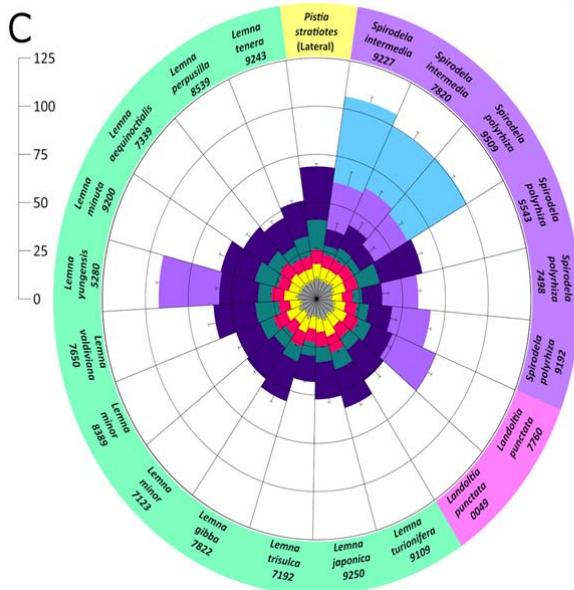
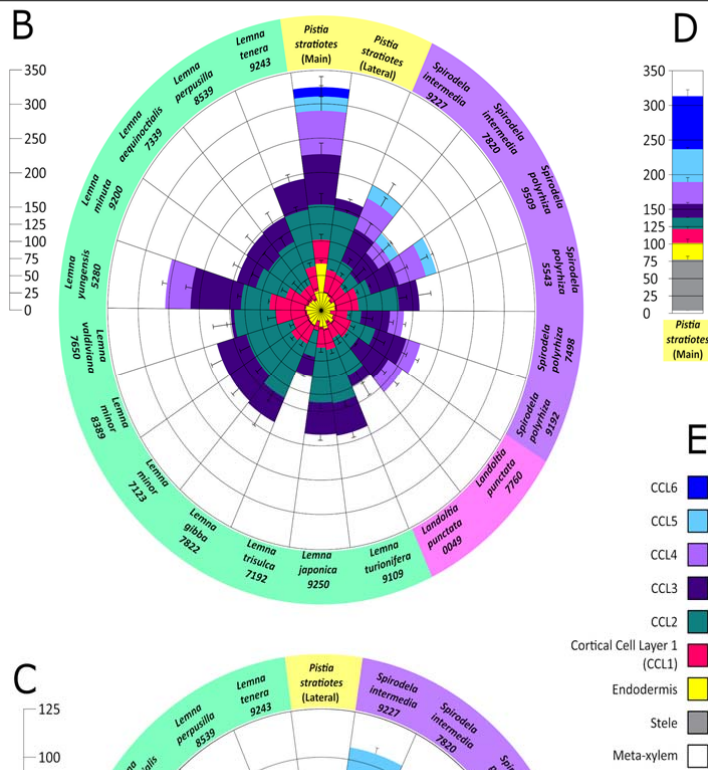
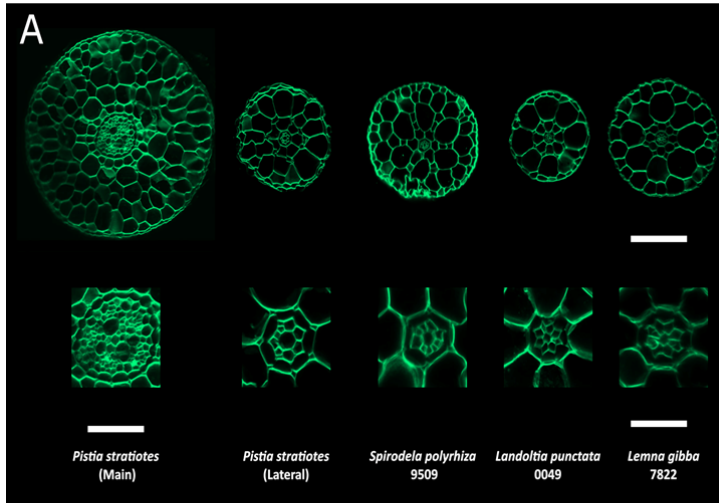
134 **Duckweed root anatomy is highly reduced**

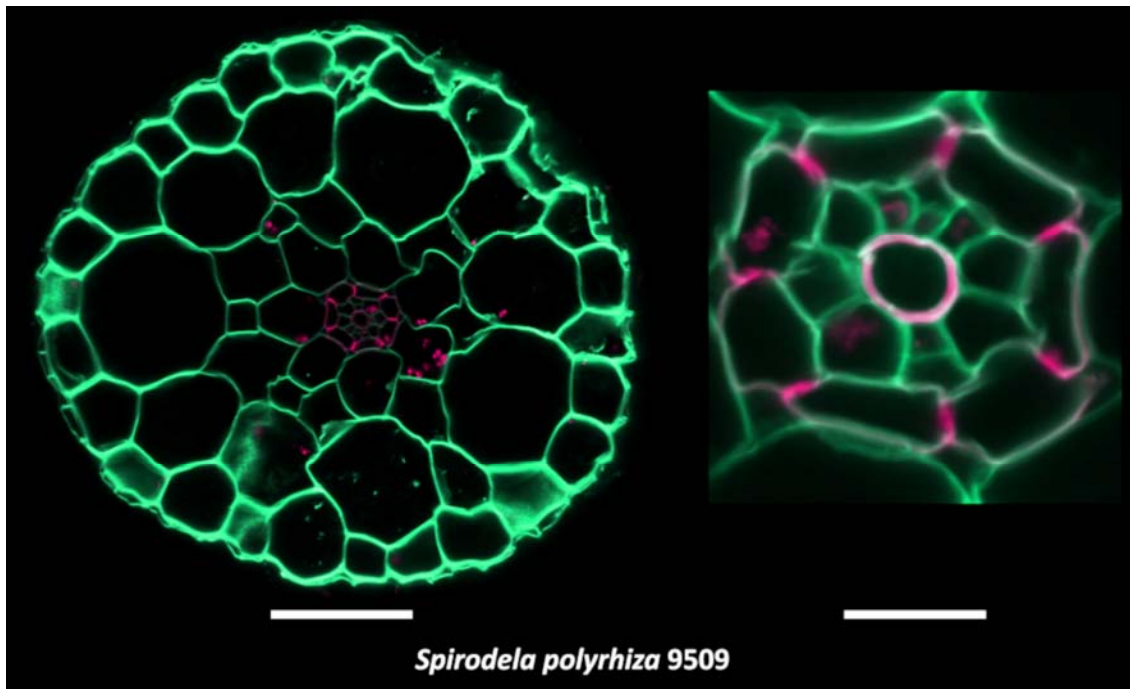
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136 Previous reports of duckweed root anatomy focused on just a few species and have not directly
137 compared these with relatives. Without outgroups, it is impossible to determine if there is a
138 trajectory towards structural reduction in duckweeds. Previous phylogenetic studies have included
139 *Pistia stratiotes* as an outgroup as another aquatic member of the *Araceae* (Les et al., 2002). *Pistia*
140 and duckweeds share several morphological and ecological similarities as free-floating macrophytes
141 but represent independent aroid lineages (Stockey et al., 1997, Wilde et al., 2005). Indeed, both
142 fossil evidence (Stockey et al., 1997, Wilde et al., 2005) and phylogenetic analyses (Friis et al., 2004)
143 suggest that duckweeds and *Pistia* independently colonized aquatic habitats, with fossils attributable
144 to the duckweeds being much older than those attributable to *Pistia* (Cabrera et al., 2008). Thus,
145 *Pistia* provides a useful model for understanding the highly reduced structure in duckweeds as its
146 form resembles ancient fossil duckweeds such as *Limnobiophyllum*.

147

148 We surveyed macroscopic root structure for 20 duckweed lines, representing 13 species, with all
149 *Spirodela* and *Landoltia* species represented and 10 of the 13 *Lemna* species, alongside *Pistia*
150 *stratiotes*. Most species were represented by multiple accessions. In no instances were lateral roots
151 or root hairs observed in duckweeds, in line with previous observations (Landolt, 1986). *Pistia* had a
152 considerably larger and more complex root system with lateral roots. 11 out of 12 duckweeds have a
153 mean root diameter between 120-200 μm , with only *Lemna yungensis* falling outside of this range of
154 means, possessing a mean diameter of 256 μm . No duckweed species possessed a maximum root
155 diameter close to the 325 μm that we observed in *Pistia*. We counted an average of 212 total cells in
156 *Pistia* cross-sections, while duckweeds display mean total cells values of 28-81 cells. *Spirodela* sp.
157 displayed mean cross section cell numbers ranging from 40 to 81 cells. *Lemna* species typically
158 displayed fewer cells in cross-section than *Spirodela*; mean values for all species are between 28 and
159 45 cells, apart from *Lemna yungensis*, which displays 73. Morphological analysis of root patterning
160 revealed a highly reduced anatomy common to all duckweed species. This consisted of a 3-5 of
161 cortical cell layers and a highly reduced vasculature (Figure 2A). All the duckweed species possessed
162 a single central xylem, typically surrounded by a small number (7-10) of what appear to be phloem
163 parenchyma cells, although this identity has never been explicitly defined. *Pistia*, conversely, has
164 multiple xylem files and considerably more phloem cells. It also has a discernible pericycle, which
165 was absent in all duckweeds surveyed here (Figure 2A, Figure 3, Supplementary Figure 2).





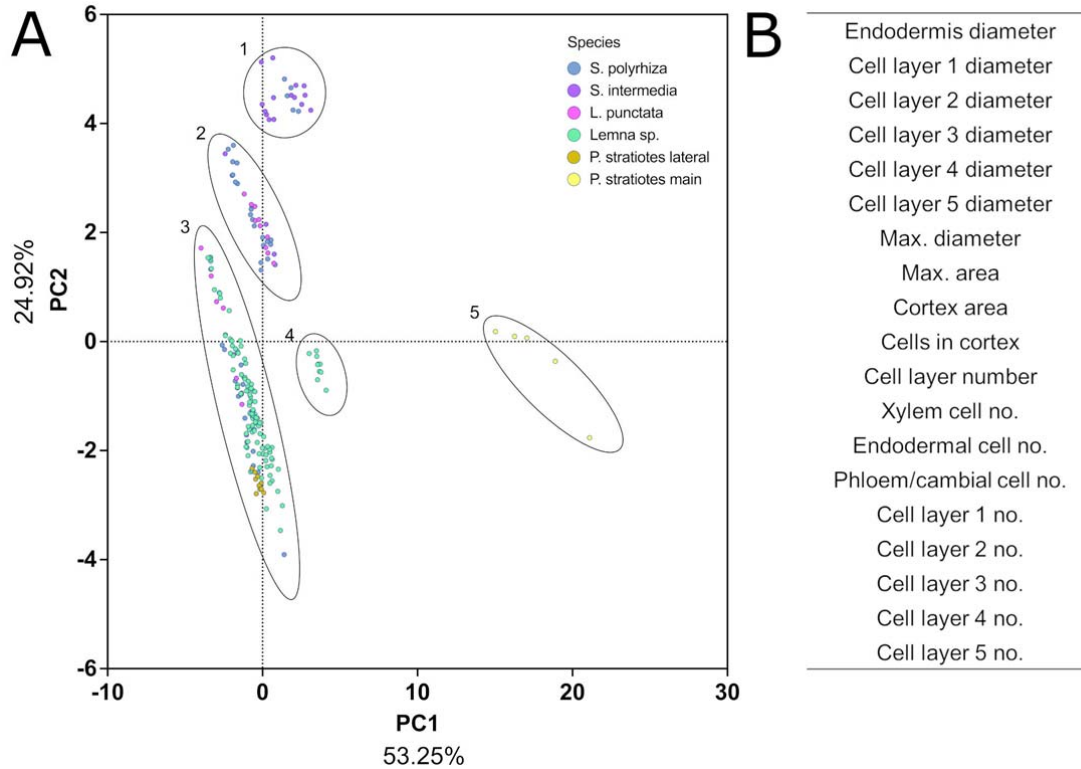
167 We observed a trend in the reduction of number of cortical cell layers (CCLs) from the earlier
168 diverging *Spirodela* (3 of 6 accessions display 5 CCLs) to the later diverging *Lemna* (3 CCLs in 11 out
169 of 12 accessions) (Figure 2B). This trend is not reflected in the root diameter (Figure 2C). Several
170 duckweed species have large extracellular air spaces within the cortex, similar to the schizogenous
171 aerenchyma found in many other aquatic plants (Jung et al., 2008). This feature appears more
172 frequently in *Spirodela* (5 out of 6 lines), in 1 out of 2 of the *Landoltia* lines, and in only 2 out of 12
173 closely related *Lemna* lines that are currently proposed to represent a single species (*yungensis*
174 *valdiviana*) (Bog et al., 2020) (Figure 2A, Supplementary Figure 1).

175

176 Compared with *Pistia*, the cell number and size of the stele and endodermis is uniformly low across
177 all duckweed species. The total number of cells enclosed by and including the endodermis is
178 remarkably invariable across duckweed species, with all duckweed species falling within the range of
179 16-18 cells, compared to approximately 100 in *Pistia*. The diameter of the endodermis is slightly
180 more variable than cell number, with the mean for all species within a range of 15-28 μm , with no
181 clear pattern between genera. The fact that duckweeds consistently showed reduced cell size and
182 number within the stele suggests reduced importance for transport within the root, consistent with
183 vestigiality.

184

185 We quantified a number of parameters relating to number and size of each cell type in the root and
186 conducted a principal coordinates analysis to survey the general trends in this anatomical dataset
187 (Figure 4). Each point represents the data captured from a root section of a separate individual



188 (Figure 4A), and the 19 variables are shown in (Figure 4B). The PCoA displays 5 distinct clusters,
 189 consistent with phylogenetic groupings. All *Lemna* species are retained in a single cluster (3), apart
 190 from *Lemna yungensis*, which forms a distinct unique cluster outside of the larger *Lemna* cluster
 191 containing this species alone (4). *Spirodela intermedia* occurs in two distinct clusters (1 and 2),
 192 neither of which contain any *Lemna* individuals. The majority of *Spirodela intermedia* individuals
 193 cluster together, in a group also consisting of a small number of *Spirodela polyrhiza* samples (cluster
 194 2). *Spirodela polyrhiza* is distributed more broadly and located within clusters 1, 2 and 3, with the
 195 majority of individuals falling into cluster 2, which contains only *Spirodela* and *Landoltia* species. A
 196 small number of *Spirodela polyrhiza* samples also fall into the *Lemna* cluster. *Landoltia* primarily co-
 197 occurs with *Spirodela polyrhiza* and *intermedia* in cluster 2, and a few individuals occur in the *Lemna*
 198 cluster. *Pistia* main roots group distant from all duckweeds driving the main axis, PC1. Interestingly,
 199 all *Pistia* lateral roots fall within the *Lemna* cluster. Given that the duckweed genera broadly cluster
 200 within their own groups, and that we see a reduction in root complexity (CCLs & aerenchyma) from
 201 *Spirodela* to *Lemna*, we propose that root anatomy is progressively reduced in more recently derived
 202 duckweed lineages.

203

204 **Continuous root removal does not reduce duckweed growth, but does reduce growth in *Pistia***
 205 ***stratiotes*.**

206

207 We hypothesised that a reduction in root complexity would be reflected by reduced requirement of
208 roots for plant growth. To test this hypothesis, we conducted root removal experiments and
209 compared the growth rate response to root removal in two representative duckweed species,
210 *Lemna minor* and *Spirodela polyrhiza*, alongside *Pistia stratiotes*. Root removal was conducted daily
211 for a period of 11 days to minimise growth of new root material. Growth (as frond or aerial tissue
212 area) was measured daily, normalised as a percent of the initial area value (Figure 5). During the
213 growth series, we observed an approximate 12 fold increase in frond area for *Lemna minor*, a 10 fold
214 increase for *Spirodela polyrhiza*, and an 8 fold increase in area for *Pistia stratiotes* for individuals in
215 control samples where roots were intact (Figure 5). For *Spirodela polyrhiza* (Figure 5A) we saw no
216 significant difference in growth for rooted versus root-excised samples. In *Lemna minor*, the only
217 significant differences in growth arose on the final three days of the growth series, where plants
218 with their roots removed displayed enhanced growth (Figure 5B). In contrast, root removal markedly
219 reduced the growth rate of *Pistia stratiotes* (Figure 5C). These results indicate that duckweed roots
220 are not required to sustain growth in laboratory conditions. These results also suggest that the root
221 is not an essential means of water absorption in duckweed.

222

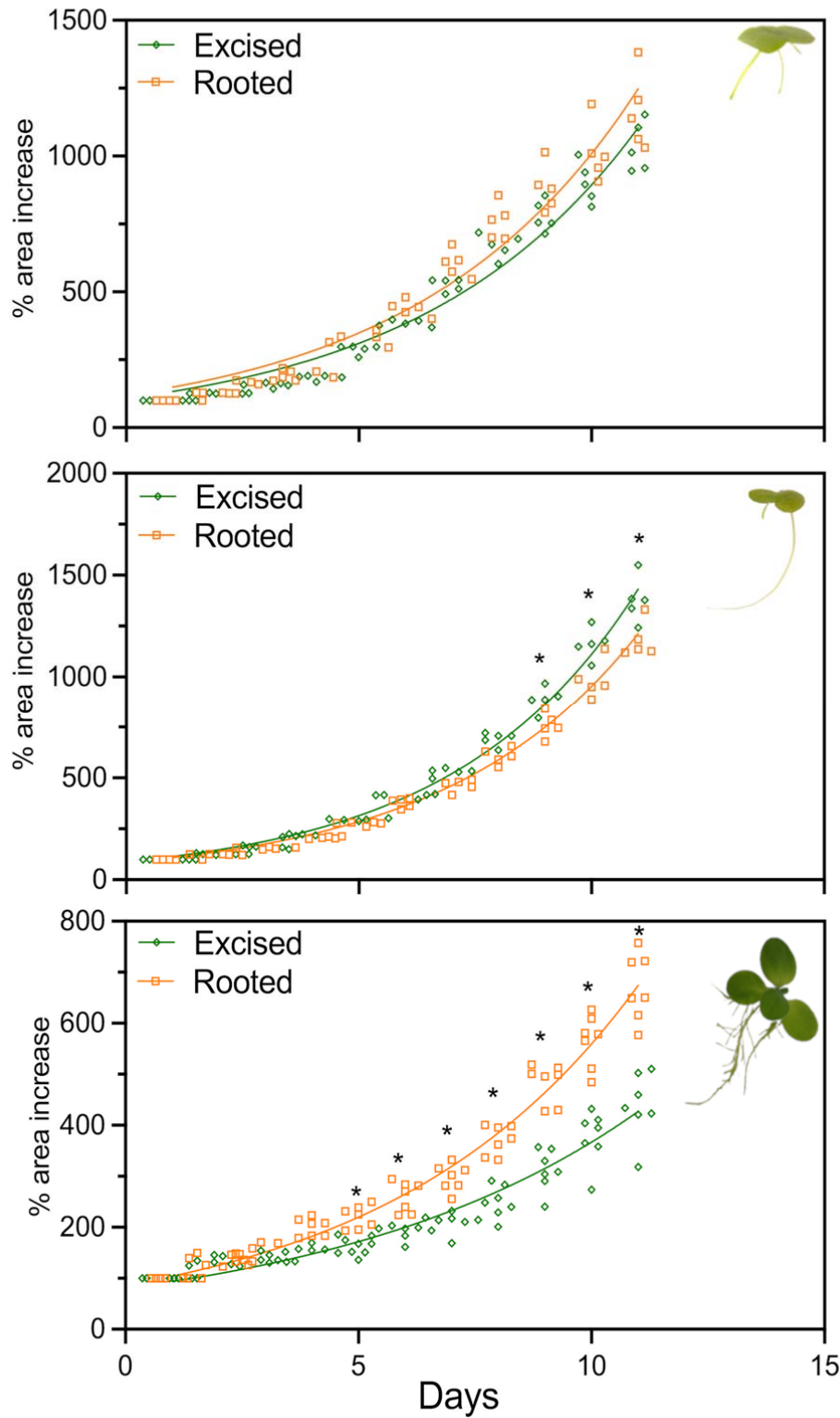
223 **Root removal does not impair the ability of *Lemna minor* or *Spirodela polyrhiza* to absorb macro-**
224 **and micronutrients, but does impact nutrient uptake in *Pistia stratiotes*.**

225

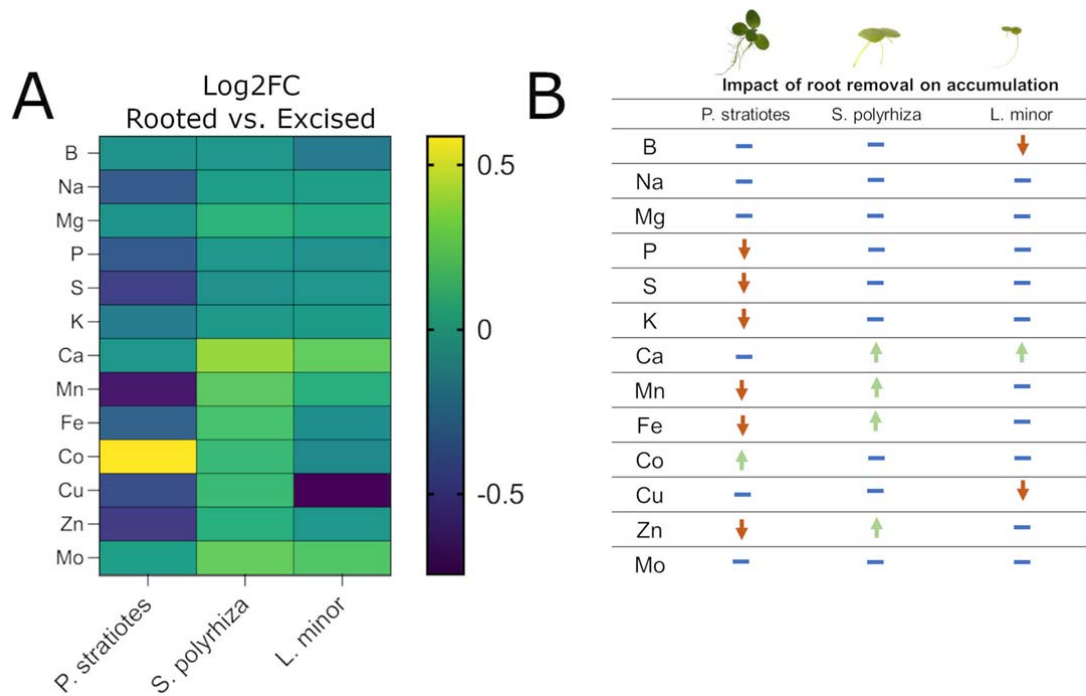
226 The growth rate assay established that rooted versus root-excised duckweeds grew in a similar
227 manner, but root removal impeded the growth of *Pistia stratoites*. We reasoned that if roots were
228 required for the uptake of specific elements, assays in which we measure specific elements would be
229 more sensitive than a crude measurement of growth in detecting the extent to which roots are still
230 required for their ancestral function. To investigate this, we subjected the fronds and aerial tissues
231 of *Pistia* generated by the previous experiment to an ionomic analysis. A total of 16 elements were
232 successfully detected in these species. Whilst some rare elements such as Li and Cd were detected,
233 we only considered 13 elements present in our growth media B, Na, Mg, P, S, K, Ca, Fe, Mn, Co, Cu,
234 Zn and Mo (supplementary figure 2). As our analysis was run under atmospheric conditions, we were
235 unable to measure the levels of N.

236

237 Root removal in duckweed made little change to the overall accumulation of nutrients in duckweed
238 (Figure 6A, B). Between *Lemna minor* and *Spirodela polyrhiza*, there were five instances where root
239 removal significantly altered elemental concentration in the frond. In three instances, root removal
240 resulted in a significantly up-regulated accumulation of certain elements: we saw increased Ca



241 concentration in the fronds of both *L. minor* and *S. polyrhiza*, and increased Fe, Zn and Mn in *S.*
242 *polyrhiza* alone. Root removal resulted in a reduction in concentration of B and Cu in *L. minor* alone



243 (Figure 6A, B, Supplementary Figure 2). In contrast, the impact of root removal on the ionic
 244 composition of *Pistia* was considerably greater, with P, S, K, Fe, Mn and Zn all being significantly
 245 reduced (Figure 6B, Supplementary Figure 2). Together, these data suggest that whilst roots are no
 246 longer required for growth and nutrient uptake in duckweeds, *Pistia* roots still play an important role
 247 in growth and nutrient acquisition. However, given that they are not absolutely required for growth,
 248 it may be that *Pistia* is *en route* to root vestigiality, albeit at a less advanced stage than the
 249 duckweeds.

250

251

252 **Discussion**

253

254 Here we sought to better resolve whether the duckweed root may be a vestigial organ, with the aim
255 of clarifying if duckweeds may serve as a helpful model for understanding the molecular
256 mechanisms underpinning organ loss. For an organ to be considered vestigial, it must have lost its
257 salient function. Typically, such organs undergo accompanying reductions in size and complexity.
258 Defining salient functions for an individual organ is challenging. However, it is clear that for almost
259 all angiosperms, a primary function of roots is to supply water and nutrients to the growing plant,
260 sustaining growth of the aboveground tissues (Boyce, 2005). We therefore examined the anatomy,
261 as well as water and nutrient uptake ability, of duckweed roots to better ascertain the position of
262 each species group along a trajectory towards vestigiality, culminating in root loss in the most
263 recently evolved *Wolffia* and *Wolffiella* (Fig. 1).

264

265 We began by surveying the anatomy of a global collection of specimens including almost all rooted
266 duckweeds, allowing us to observe if a) the reduced anatomy in duckweeds is consistent between
267 species and genera and b) if any trends in root reduction are present at the anatomical level. This
268 built upon previous reports looking into a handful of species (An et al., 2019; Landolt, 1986;
269 Melaragno and Walsh, 1976), expanding it considerably to encompass almost all rooted species of
270 duckweeds. We compared duckweed root morphology with the sister *Pistia stratoites*, which is
271 believed to have undergone an independent and more recent invasion of the aquatic environment.
272 Our findings revealed that duckweed roots are consistently reduced in both size (diameter) and
273 morphological complexity compared with *Pistia*, consistent with the idea that they are no longer
274 required for active nutrient transport (Figure 2A, Supplementary Figure 2).

275

276 As well as the macroscopic reduction in root system complexity - multiple roots per frond to single
277 root per frond - in *Spirodela* and *Landoltia* versus *Lemna*, we also leveraged our anatomical data to
278 question whether root anatomical complexity reduces concurrently with root number. We observed
279 a reduction in both the number of cortical cell layers and the presence of aerenchyma between
280 *Spirodela* spp. and *Lemna* spp. The apparent decrease in complexity between *Spirodela* spp. and
281 *Lemna* spp. supports a model in which traits associated with root complexity have been
282 progressively lost in duckweeds as novel species have formed, accompanying the reduction in root
283 number. In comparison, *Pistia* plants may be less far along this trajectory towards root
284 vestigialization. A PCoA encompassing all root anatomical traits measured further confirmed these
285 observations. Virtually all individuals of the genera *Spirodela* and *Landoltia* sit in two distinct clusters

286 based on their root anatomy, separated from *Lemna* individuals, which exist almost exclusively in a
287 single cluster, matching their monophyletic origin. This correlation with phylogenetic groupings
288 further supports the concept that root anatomy has evolved to become further reduced in *Lemna*.
289 We feel root loss in duckweed presents a unique opportunity for deepening our understanding of
290 vestigiality. In other models of organ loss, such as cavefish, evolution has produced a more binary
291 range of traits (i.e. sighted versus unsighted fish). In comparison the duckweed root offers a greater
292 spectrum of phenotypes in terms of both root number and anatomy, providing a rich pool of
293 germplasm within which we can explore networks controlling discrete aspects of root development.

294

295 The anatomy of the duckweed root is also highly similar to that of lateral roots in *Pistia*. This cellular
296 arrangement is similar to that of fine lateral roots of other monocot species (Watanabe et al., 2020).
297 When root anatomical trait values are mapped onto a PCoA, *Pistia* lateral roots sit in a cluster which
298 is primarily composed of *Lemna spp.* It is feasible that this cellular arrangement seen in *Lemna*
299 represents or is approaching an anatomical ‘minimum’ without which it would not be possible to
300 form a root.

301

302 If duckweed roots are vestigial, they should not only have reduced complexity but will have lost
303 some or all of their salient function. We showed that whilst *Pistia* roots had a positive and significant
304 effect on leaf growth, growth of duckweed fronds was largely unaffected in rooted versus rootless
305 samples, implying that roots are dispensable for providing nutrients and water for growth. Growth
306 data alone do not provide a full picture of capacity for nutrient transport. We therefore leveraged an
307 ionomics platform that permitted a survey of the elemental landscape of duckweed fronds when
308 grown without a root, which we compared with *Pistia stratiotes*. We considered a broad suite of
309 nutrients including every element present in our growth media, except nitrogen. We did not see
310 major shifts in the elemental composition of the fronds of either duckweed species when subjected
311 to continuous root removal. Strikingly, no elements included in our analysis (0 out of 13) exhibited
312 reduced accumulation in *Spirodela polyrhiza* grown without roots, and only 2 out of 13, B and Cu,
313 did in *Lemna minor*. Conversely, in *Pistia stratiotes*, 6 of the 13 elements quantified exhibited
314 reduced accumulation in shoot tissues as a consequence of root removal, including elements critical
315 for growth with well-established root-mediated uptake mechanisms such as P and K. Together, this
316 clearly evidences the dispensability of roots in duckweeds for nutrient uptake. A surprising result in
317 both *Spirodela* and *Lemna* was the increase in certain nutrients following root excision. This included
318 Ca, Fe, Zn, and Mn, with Ca being consistently elevated. A potential hypothesis is that duckweed
319 roots could be repurposed for the storage or sequestration of nutrients. Raphides (calcium oxalate

320 crystals) are present in *Lemna minor* and have been shown to localise within roots (Franceschi 1987,
321 1989).

322

323 Considering the definitions of vestigiality by both Prout and Muller, we feel that these data clarify
324 that duckweed roots are indeed vestigial, and to varying degrees across the group, opening the door
325 to their utilisation as models for understanding this vestigiality. This gradient also poses a key
326 question. If duckweed roots are vestigial, why are they maintained in some species? Whilst some
327 vestigial structures may be non-functional, others may have gained novel functions as a
328 consequence of reduced constraint (i.e., exaptation), whilst other structures may be in an
329 intermediate state whereby the transition to vestigiality is incomplete (Walker-Larsen and Harder,
330 2001). It is therefore possible that relaxed selection pressure has permitted duckweed roots to
331 become neofunctionalised to perform novel roles. It has been suggested that duckweed roots may
332 function as organs of stability (Landolt, 1986) or aid dispersal by adhering to animals (Cross, 2017).

333

334 In conclusion, these results support a model of progressive vestigiality of roots across the
335 duckweeds. Broadly it points to a duckweed root that is both anatomically simplified and
336 dispensable for the salient functions of water or nutrient uptake. However, we acknowledge that
337 our experiments do not completely rule out a role for root in nutrient uptake under, for example,
338 limiting conditions or in natural habitats, replete with companion species and competitors. However,
339 these results lay a foundation for the use of duckweed roots as a model system for further
340 investigation into the molecular and evolutionary processes underlying vestigiality in plants.

341

342

343 **Methods**

344

345 **Duckweed growth and culture**

346

347 All duckweed stocks employed in this experiment were obtained from the Landolt collection, ETH
348 Zurich (<http://www.duckweed.ch>), except for Spirodela polyrhiza lines 9509 and 7948 which were
349 provided by Klaus Appenroth, Friedrich Schiller University, Jena. Four-digit numerical codes following
350 species names refer to their Landolt accession number. Stocks were maintained on liquid N-media or
351 SH-media (Appenroth et al., 1996) at 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light and 16/8h light cycle in a Conviron
352 growth chamber, set to 22°C with 70% RH. *Pistia stratiotes* was obtained from JAM Aquatics,
353 Wrexham, UK.

354

355 **Root cross section anatomy**

356

357 Plants were grown in 250 ml conical flasks containing 150 ml of liquid N-media in the same
358 conditions as stocks. Flasks were inoculated with 5-10 colonies from the stock collections and grown
359 for 2-6 weeks. Plants selected possessed roots of average or greater length, and fronds of average or
360 greater area based on visual appraisal.

361

362 Vibratome sectioning of duckweed roots was conducted as per Jones et al., (2021). For each line, ten
363 individual plants per line were embedded and sectioned, and 5-10 root sections were collected per
364 plant, stained using the method described in Atkinson and Wells (2017), and imaged using confocal
365 laser scanning microscopy. Basic fuchsin staining was conducted at a concentration of 0.01%
366 following sectioning. A single image section per plant was selected based on quality and
367 representation, then measured using FIJI (Schindelin et al., 2012). Cells were classified into layers in
368 concentric rings from the endodermis outwards. The diameter of each layer was measured, as was
369 the number of cells in each layer, along with the diameter of the endodermis, number of
370 endodermal cells, and number of cells in the stele. Diameters were measured using the ruler tool. At
371 each layer, diameter was measured from 5 points around the circumference of the layer, measuring
372 the maximum distance between points on the layer, then the mean was taken of these 5 points for
373 each layer. Epidermal cells had poor dye penetration, and a reduced fluorescence on the confocal
374 microscope, and so could not be reliably counted.

375

376 **Root removal treatments and imaging**

377

378 For the root removal experiment, plants were grown in Schenck-Hildebrandt (SH) media. For the
379 control treatment, no manipulation was undertaken. In the root removal treatment, all visible roots
380 were removed from colonies daily using ethanol sterilised surgical scissors. For *Spirodela polyrhiza*
381 and *Lemna minor*, each treatment consisted of five individual flasks, each seeded with 3 colonies
382 onto 100 ml of media. Individual flasks were treated as a replicate and flasks were arranged
383 randomly in the growth cabinet and re-randomized daily. For *Pistia stratiotes*, each flask was seeded
384 with a young individual plant with 3 emerged leaves visible to the naked eye, to a total of 7
385 plants/treatment. The treatment regimen was conducted for 11 consecutive days.

386

387 Plants were imaged daily in their flasks from beneath, utilising a transparent raised platform
388 featuring a water bath in which to place the flasks to correct for the optical distortion. Images were
389 processed using FIJI to measure frond or aerial tissue area. For duckweed flasks, RGB images were
390 split into their constitutive 8-bit channels, and the blue channel retained. Frond tissues alone were
391 then selected using the threshold tool and area measured. For *Pistia*, images were again split, but
392 the red channel retained. This was then subject to gaussian blur (sigma = 7.0) and again only the
393 aerial tissues selected using the threshold tool. In rooted samples where this alone was not
394 sufficient to separate frond and root, the select polygons tool was used to exclude any additional
395 root captured by thresholding.

396

397 **Ionic analysis**

398

399 Samples were harvested immediately following the root removal experiment. Prior to harvesting,
400 roots were removed from fronds or aerial tissues and washed 3 times for 2 minutes with MilliQ
401 water. Samples were placed in pre-weighed Pyrex test tubes, and dried at 88°C for 24h. Then, dry
402 weight was recorded, and 1 ml concentrated trace metal grade nitric acid Primar Plus (Fisher
403 Chemicals) spiked with in internal standard was added to the samples that were further digested in
404 DigiPREP MS dry block heaters (SCP Science; QMX Laboratories) for 4 hours at 115°C following the
405 method adapted from Danku et al.,2013. After digestion, samples were diluted to 10 mL with 18.2
406 MΩcm Milli-Q Direct water and elemental analysis was performed using an ICP-MS, PerkinElmer
407 NexION 2000 and twenty-three elements were monitored (Li, B, Na, Mg, P, S, K, Ca, Ti, Cr, Mn, Fe,
408 Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd and Pb). To correct for variation within ICP-MS analysis run,
409 liquid reference material was prepared using pooled digested samples, and run after every nine
410 samples. Sample concentrations were calculated using external calibration method within the

411 instrument software. Further data processing including calculation of final elements concentrations
412 was performed in Microsoft Excel.

413

414 **Statistical analyses**

415

416 All statistical analyses were conducted in GraphPad Prism version 9.0 (graphpad.com). For the
417 anatomical dataset, principal coordinates analysis was conducted on 19 variables and 210 rows
418 utilising parallel analysis with 1000 simulations and a random seed. For root removal experiments,
419 two-way repeated measures ANOVA was performed, followed with Sidaks' multiple comparisons
420 test to establish differences in growth on a per-day basis. For nutrient concentration comparisons
421 generated by ionomic analyses, data were compared with one-way ANOVA followed by Sidak's
422 multiple comparison's test to establish differences in concentration between individual nutrients.
423 Log₂ fold changes generated from ionomic data were calculated as Log₂(elemental conc. roots
424 removed)-Log₂(elemental conc. rooted).

425

426 **Figure legends**

427

428 Figure 1. Representative phylogeny of the duckweed genera and *Pistia* highlighting the progressive
429 loss of roots of roots and loss of individual root traits as genera diverge (indicated by + and -; arrows
430 next to cortical cell layers indicate the progressive reduction in layer number as the genera diverge)
431 (after Tippery and Les, 2020). Representative images (not to scale) of species from each genera are
432 shown for illustrative purposes.

433

434 Figure 2. Comparison of root anatomical traits across almost all extant duckweeds reveals a highly
435 reduced anatomy. A) Representative images of root sections from species representing each
436 duckweed genera and main and lateral roots of *Pistia stratiotes*. Images were obtained via fresh
437 tissue sectioning and confocal imaging. Scale bar = 50 μ M for entire roots; 10 μ M for vasculature
438 close-up. B) Rose diagram displaying the width of each cell layer (μ m) for roots of 20 duckweed lines
439 encompassing 13 species, denoted at the outside of the circle. C) Rose diagram displaying the
440 number of cells in cell layer for roots of the aforementioned lines, denoted at the outside of the
441 circle, with *P. stratiotes* main roots (D) in a separate bar chart for ease of resolution. Background
442 colour underlying the species labels represents genera; yellow represents *Pistia*, purple *Spirodela*,
443 pink *Landoltia*, green *Lemna*. E) Colour coded key to the different cell layers displayed on the rose

444 diagrams. CCL stands for cortical cell layer. $n = 10$ root sections derived from different plants, except
445 for *Pistia stratiotes* (main) and *L. trisulca* where $n = 5$ root sections derived from individual plants.

446

447 Figure 3. Basic fuchsin staining of duckweed vasculature highlights lignification in the endodermis
448 and central xylem. Entire root section and accompanying close up of the vasculature of *Spirodela*
449 *polyrhiza* 9509 with cell wall staining (calcofluor white; green) and lignin staining (0.01% Basic
450 Fuchsin; magenta). Scale bar = 50 μM for entire roots; 10 μM for vasculature close-up.

451

452 Figure 4. Principal coordinates analysis of duckweed anatomical traits highlights interspecies
453 differences and a gradient of reducing root anatomical complexity. A) PCoA based on 21
454 components, with 210 rows, derived from an anatomical analysis of fresh root sections from 20
455 duckweed lines, encompassing 13 species, and main and lateral roots of *Pistia stratiotes*. Clusters
456 have been manually highlighted and numbered for ease of further discussion. Percentage of
457 variance explained by each PC is indicated on the relevant axis. B) Summary of the 19 variables used
458 to generate the PCoA in A.

459

460 Figure 5. Growth of the duckweeds *Spirodela polyrhiza* and *Lemna minor* is not impacted by
461 continual root removal, unlike the aroid *Pistia stratiotes*. Plants were subjected to continuous root
462 removal and growth compared to untreated controls. Growth was measured as area of fronds (or
463 aerial tissues for *Pistia*), derived from daily imaging from beneath, and plotted as a percentage
464 increase relative to the initial (day 1) area value. Lines show the best fit of an exponential growth
465 curve. A) *Spirodela polyrhiza*; B) *Lemna minor*; C) *Pistia stratiotes*. $n = 5$ flasks, each initially seeded
466 with 3 colonies for duckweeds; $n = 7$ flasks, each initially seeded with 1 plant for *Pistia*. Asterisks
467 show statistically significant differences as assessed by two-way repeated measures ANOVA
468 followed by Sidak's multiple comparisons. Lines show the best fit of an exponential (Malthusian)
469 growth curve.

470

471 Figure 6. Continuous root removal has a limited effect on element accumulation on the duckweeds
472 *Spirodela polyrhiza* and *Lemna minor* but reduces the accumulation of a number of elements in the
473 aroid *Pistia stratiotes*. A) Heatmap showing the log₂ fold change of rooted versus rooted elements
474 for each species. B) Table synthesising the data generated in A) indicating whether root removal
475 results in statistically significant increased accumulation (green upwards arrow), decreased
476 accumulation (red downwards arrow), or no significant change (blue hyphen). Significance ($P < 0.05$)
477 was determined by one-way ANOVA followed by Sidak's multiple comparisons test. $n = 5$ flasks, each

478 initially seeded with 3 colonies for duckweeds; $n = 7$ flasks, each initially seeded with 1 plant for

479 *Pistia*.

480

481

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